GENETIC BASIS OF NITROGEN USE EFFICIENCY AND ITS APPLICATION IN WHEAT BREEDING

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Abstract:

Nitrogen is the major limiting factor in crop production worldwide, and N is now the single largest input cost for many crops including wheat. Nitrogen use efficiency (NUE) is a complex trait, and as many as 25 genes have been estimated to be involved in regulating NUE in the diploid model species Arabidopsis. Numerous quantitative trait loci (QTLs) for agronomic traits associated with N use and yield have been mapped in wheat, but no gene has been characterized in this crop species. The genetic mechanisms controlling NUE in wheat are unknown.

In this study, we cloned a major QTL for N-related agronomic traits in winter wheat using map-based cloning approach. Based on the phenotypes and genotypes of critical recombinant lines that were generated from a cross between two winter wheat cultivars, 'Jagger' and '2174', we delimited the QTL into a genomic region including three candidate genes. We found that the vernalization gene *TaVRN-A1* was tightly linked with *TaNUE1*, the gene shown to influence NUE in wheat. Because of an Ala^{180}/Val^{180} substitution, *Ta*VRN-A1a protein encoded by the Jagger allele and *Ta*VRN-A1b protein encoded by the 2174 allele interacted differentially with *Ta*ANR1, a protein encoded by a wheat orthologue of *Arabidopsis nitrate regulated 1* (*ANR1*). The transcripts of both *TaVRN-A1* and *TaANR1* were down-regulated by nitrogen. *TaANR1* was functionally characterized in *TaANR1::RNAi* transgenic wheat and in a natural mutant with a 23-bp deletion including 10-bp at the 5′ end of intron 5 and 13-bp of exon 6 in its gDNA sequence, which produced transcript that lacked the full 84-bp of exon 6. Both *Ta*ANR1 and *Ta*HOX1 bound to the Ala^{180}/Val^{180} position of *TaVRN-A1*. These findings explained why NUE was regulated by *TaVRN-A1* gene in wheat.

We genotyped *TaVRN-A1*, *TaANR1* and *TaHOX1* and characterized grain yields in the Jagger x 2174 recombinant inbred lines in a field for two years. Genetically incorporating favorable alleles from *TaVRN-A1*, *TaANR1* and *TaHOX1* increased grain yield by 9.84% to 11.58% in the field. Molecular markers for allelic variation in these three genes can be used in breeding programs aimed an improved NUE and grain yield.

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CHAPTER I

GENERAL INTRODUCTION

1.1 Wheat as an important crop

Wheat (*Triticum aestivum L*. 2n=6x=42, AABBDD) is one of the most important crops for human nutrition and animal feed in the world. U.S wheat provides approximately 11% of the world supply, and nearly 35% of world exports in recent years (McMichael, 2001). Due to the increasing world population from current 7.5 billion to 9 billion by 2050, high productivity of wheat is required to face the potential food shortage (Tilman et al., 2001).

Wheat is traditionally classified into winter or spring wheat. Winter wheat requires a long period at low temperatures during the winter season before flowering, called vernalization, which will accelerate the transition process from vegetative to reproductive development. Spring wheat does not have a vernalization requirement (Pugsley, 1971). In 2015-2016, 5.3 million acres of winter wheat were planted in Oklahoma accounting for 14.6% of total winter wheat acres planted in the USA, which were 36.2 million acres (USDA National Agricultural Statistics Service). Approximately 70% of winter wheat

fields in Oklahoma are used for winter forage production such as grazing, hay or silage (Zhang et al., 2004), and about 49 % of the wheat fields are managed for dual-purpose for both grazing and grain production (Hossain et al., 2004).

1.2 Nitrogen (N) as essential nutrient related to crop yield

Application of N fertilizer for improving crop yield

Nitrogen is an essential nutrient for plant development and growth. It is a major component of chlorophyll used by plants to produce sugars by capturing energy from sunlight during the process of photosynthesis. It is also a major component of amino acids, the building blocks of protein, nucleotides, and other metabolites and cellular components, which are used during the whole life of the plant (Kong et al., 2013; Lemoine, 2013). However, most plants cannot directly utilize the dinitrogen gas (N_2), which constitutes approximately 78% of the atmosphere. Thus, application of N fertilizer is an effective approach to improve the cropping system in agriculture, which results in higher grain and protein yield. N fertilizer can provide plants two forms of N including ammonium (NH_4^+) and nitrate (NO_3^-) in the soil, which can be absorbed through plants' root system (Santi et al., 2013). It is often supplemented to non-N-fixing food crops such as wheat, rice (*Oryza sativa L.*) and maize (*Zea mays L.*) to ensure the harvest of high grain yields (Santi et al., 2013). A sevenfold increase in the use of N fertilizer was found to be associated with a twofold increase in food production over the past four decades (Hirel et al., 2007; Shrawat et al., 2008).

Adverse effects of N fertilizer on the environment

Although N fertilizer has the most direct and efficient approach for increasing crop production, the synthetic N fertilizers supplied to soils have immediate and adverse effects on

the environment and climate. Only 30%–35% of added N fertilizers are taken up and used by wheat plants in the year of application, and the remaining 65%–70% (assuming fertilizer–soil equilibrium) is lost, which contributes to atmospheric greenhouse gases and environmental pollution (Gaju et al., 2011; Raun and Johnson, 1999).

The potential pollution from using the excess amount of N fertilizers is predominately caused by nitrate leaching, denitrification, and volatilization (Tilman, 1999). Nitrate leaching contributes to NO_3^- contamination of soil, groundwater and oceans. Nitrate is motile in the soil, which is easily lost in the root environment with water percolating through the soil in high rainfall or irrigated conditions. When high rates of N fertilizers are applied to agricultural fields, excess NO_3^- will be leached into the water system causing eutrophication of the freshwater (London, 2005) and marine ecosystems (Beman, et al, 2005; Hirel, et al., 2007). Denitrification and volatilization contributes to the gaseous emissions of N oxides reacting with the stratospheric ozone and the emission of toxic ammonia into the atmosphere (Ramos, 1996; Stulen et al., 1998). Microbes facilitate denitrification, NO_3^- is reduced, and ultimately produces N_2 with intermediate gaseous nitrogen oxide products during denitrification. When the water-logged soil become anaerobic and favorable for microbial activity, some microorganisms will strip the oxygen from NO_3^- and release the gaseous form of nitrogen, including nitrous oxide (N_2O) and nitrogen gas (N_2). N_2O is a major greenhouse gas emission, and the N_2O emission originating from using N fertilizer is estimated to account for more than 70% of the total N_2O emissions (Mortimer et al., 2004). The volatilization usually happens in high pH soils when N is in the organic form of urea, most commonly from animal manures or urea fertilizers. It will change N to ammonia gas (NH_3), which is lost into the atmosphere when soil is warm and the source of urea is on the soil

surface. Ammonia is the precursor for developing the potentially harmful microscopic particulate matter associated with $PM_{2.5}$ and PM_{10} particulate air pollution (Mensink, 2008), which has potential negative effect for the environment and human health.

The requirement of N fertilizer for dual-purpose winter wheat

Winter wheat requires significantly more N to achieve maximum grain compared with spring wheat because of the longer growing season and potential N loss (Goos and Johnston, 1999). In the southern Great Plains, dual-purpose winter wheat cultivars are grown with more flexibility and additional economic advantages compared with forage-only or grain-only wheat. They require additional N fertilizer due to N removal in consumed forage during winter compared with grain-only wheat (MacKown and Carver, 2007). Thus, varieties able to maintain yield under lower N conditions are desirable for a productive dual-purpose winter cropping system and an environmentally sustainable agriculture.

1.3 Understanding N response pathways

Plants take up N primarily as NO_3^- and NH_4^+, with NO_3^- being the predominant form in most agricultural soils (Crawford and Forde, 2002). The response of plants to added N involves genes and enzymes in the following pathways, including acquisition, assimilation, translocation, as well as remobilization of N within the plants. Important genes and enzymes involved in these pathways have already been identified in Arabidopsis (Krapp, 2015).

Acquisition and transportation of N in plant

The first step of N acquisition by the root is active transportation across the plasma membrane of root epidermal and cortical cells. Plant roots have uptake systems with different

affinities for acquiring NO_3^- and NH_4^+ under variant concentrations ranging from lower than 100 μM to higher than 10 mM in soil solutions. Nitrate uptake systems have been traditionally classified into two physiological groups: the high-affinity transport system (HATS) and the low-affinity transport system (LATS) (Crawford 1995). The HATS are further subdivided into constitutive (CHATS) and inducible (IHATS) systems (Aslam et al., 1993). The LATS are involved in NO_3^- uptake at high external concentrations of NO_3^- (>0.2 mM), while the CHATS and IHATS are saturated at low external NO_3^- concentrations (approximately 100 μM). Genes encoding proteins for NO_3^- transportation, which are nitrate transporters (NRT), in these two systems have been defined in Arabidopsis. Most of them fall into two families: *NRT1* for LATS and *NRT2* (or *NAR2/NRT2*) for HATS. One exception for *NRT1* family is *NRT1.1* (*CHL1*), which can have function over both ranges of concentration (Xu et al., 2012). Ammonium is mainly taken up via plasma membrane located ammonium transporter (AMT), which can transport either hydrophobic NH_3 or charged NH_4^+ (Xu et al., 2012).

Assimilation and remobilization of N in plant

In most plants, some NO_3^- can be metabolized directly in the root after uptake. However, the larger part will be transferred to the aerial parts of the plants. In many species, NO_3^- assimilation happens in the shoot (Andrews, 1986) where NO_3^- is first reduced in the cytoplasm to nitrite by nitrate reductase (NR) with a side product of nitrogen oxide (NO), and then transported into the chloroplast by HPP transporters and reduced to NH_4^+ by nitrite reductase (NiR).

Ammonium from reduced NO_3^- or directly uptake N by AMTs from the soil is assimilated

into glutamate (Glu) via the glutamine synthetase (GS)/glutamine-2-oxoglutarate aminotransferase (GOGAT) cycle. There are predominantly two types of GS/GOGAT isoenzymes in higher plants. The chloroplastic GS2 and Fd-GOGAT enzyme are essential for assimilation of NH_4^+ from photorespiration (Xu et al., 2012). The NH_4^+ imported from the environment can be assimilated by the cytosolic GS1/NADH-GOGAT enzyme. GS1 isoenzymes have the function in different plant development processes with various affinities for NH_4^+ (Thomsen et al., 2014). NADH-GOGAT takes part in the non-photorespiratory pathway and also assimilates NH_4^+ in the root (Kojim et al., 2014). Asparagine (Asn) synthetase, together with GS, is essential for N assimilation within the plant (Gaufichon L et al., 2013), which catalyzes the formation of Asn and glutamate (Glu) from glutamine (Gln) and aspartate (Asp). Then, the Glu amino group can be transferred to amino acids by a number of different aminotransferases and these amino acids will be further used in protein synthesis (Lam et al., 1995). In vegetative stage, the N in the form of protein is stored in leaves that are a sink for N. Later, during senescence, this N is remobilized to seeds mainly as amino acids by degrading protein in the senescence leaves, which constitutes 95% of the seed protein (Okumoto S and Pilot G, 2011; Taylor L et al., 2010; Xu et al., 2012). Chloroplasts have been shown providing the main source of nutrients during senescence.

Regulation of NO_3^- as a signal in N assimilation pathways

Nitrate is not only the main source of N for plants, but also acts as an important signal for several developmental processes. It triggers changes in expression of nitrate-regulated genes and its concentration affects lateral root development, root architecture and the root-to-shoot ratio (Kant et al., 2011). For example, *AtNRT1.1* (*CHL1*) was known as a dual-affinity nitrate transporter. It also functioned as a nitrate sensor triggering the nitrate-specific

signaling pathway and linking NO_3^- and lateral root proliferation by acting upstream of *Arabidopsis nitrate regulate 1* (*ANR1*) (Remans et al., 2006). *AtANR1* was reported as a MADS-box transcription factor gene regulating localized lateral root growth in response to NO_3^- (Zhang and Forde, 1998).

1.4 Concepts of nitrogen use efficiency

The most used definition of nitrogen use efficiency (NUE) among breeders is defined by Moll et al. (1982), which is the grain dry mass (Gw) divided by the total N supplied to plants. It is divided into two components: nitrogen uptake efficiency (NUpE) and nitrogen utilization efficiency (NUtE). NUpE is the efficiency of absorption/uptake of supplied N, which is calculated as the total amount of N in the above-ground plant at harvest (Nt) divided by the available N supplied to plants from soil and fertilizer (Ns). It explains the capacity of plant roots to acquire N from the soil (Xu et al., 2012). NUtE is the efficiency of assimilation and remobilization of plant N to ultimately produce grain (Good et al., 2004; Han et al, 2015), which is calculated as the Gw divided by Nt. It is the fraction of plant-acquired N to be converted to total plant biomass or grain yield.

The total N uptake from the soil is affected by the developmental stage of the plant. Harper et al. (1987) found that maximum N accumulation had occurred at or near flowering in wheat and corn, not at harvest. Thus, the N accumulation at different stages needs to be considered for some crops when evaluating NUpE. Also, the calculation of the efficiency of the soil supplying N to plants is complicated, which is strongly influenced by immobilization and mineralization under changing climates and environments. Calculating the available N to the crop is complicated by a number of factors, including residual N in the soil at sowing, N loss

during aerial N deposition, inorganic forms of N gain from the mineralization of the organic matter and available N from applied fertilizers. In some studies, the N available from soil has been estimated as the combined NO_3^- and NH_4^+ content of the soil to a defined depth (e.g. 90 cm) prior to sowing and N-fertilizer application (Muurinen et al., 2006; Anbessa et al., 2009). This method ignores the contribution of N released by mineralization of organic matter. In other studies, N available to the crop was to be equivalent to the above-ground crop N content at harvest in control plots without fertilizer-N (N_{off} zero fertilizer) (Sylvester-Bradley and Kindred 2009) or above-ground crop N content at harvest from control plots without fertilizer-N plus the residual NO_3^- and NH_4^+ content of the soil at harvest (Huggins and Pan, 1993; Paponov et al., 1996). Bingham et al. (2012) compared these methods to estimate residue N from the soil and showed no significant difference between these calculations for the ranking of NUE for different barley genotypes. Thus, the simplest method could be used to calculate the N available for the crop.

1.5 Contribution of physiological plant traits to NUE

The studies for physiological mechanisms for increasing NUE mainly focus on two parts: the mechanisms for traits influencing NUpE and NUtE.

Physiological traits related to NUpE

Physiological traits increasing NUpE are related to root architecture and root's ability to capture available N from the soil. Improving traits for root mass and depth, root axis length, and lateral branching will maximize N capture from the soil (Foulkes et al., 2009). Ammonium and NO_3^- are main sources of N for plants to uptake from the soil through an active transport system by ammonium and nitrate transporters in the root cell plasma

membrane (Loque and von Wiren, 2004; Glass, 2009). Increasing the activity of nitrate and ammonium transporter may increase NH_4^+ and NO_3^- uptake efficiency from the soil. For example, *TaNFYA-B1*, a subunit of nuclear factor Y, stimulated root development and up-regulated the expression of the genes encoding nitrate transporters in roots, resulting in a significant increase in N uptake and grain yield (Qu et al., 2015). In wheat, *TaNAC2-5A*, a transcription factor-encoding a NAC (NAM, ATAF, and CUC), was found to bind to the promoter region of the genes encoding nitrate transporter and glutamine synthetase, resulting an enhanced ability of roots to acquire N and increased grain yield (He et al., 2015). Studies on genes related to nitrate signaling and root proliferation in Arabidopsis also provide prospects to modify the root morphological system to increase NUpE (Zhang et al., 1999; Foulkes et al., 2009). One recent study has already shown the activity of nitrate transporter affects NUE. For example, *NRT1.1B* was responsible for a higher NUE identified in rice (Hu et al., 2015). *NRT1.1* also facilitated the uptake of auxin response to NO_3^-, affected the root branching, and activated the *ANR1*-dependent signaling pathway that stimulated lateral root elongation (Mounier et al., 2014).

Physiological traits related to NUtE

Physiological pathways for improving NUtE may include ones in N assimilation and N remobilization from senescent tissues. GS, a key enzyme in N assimilation, played an essential role in N remobilization, growth rate, yield, and grain filling (Bernard and Habash, 2009). A study showed that GS activity had a positive relationship with NUtE and grain yield in maize (Masclaux et al., 2001). The post-anthesis N remobilization was increased under high activity of GS and other key N assimilation enzymes (Foulkes et al., 2009). A glutamate synthase (GOGAT) gene on wheat chromosome 3B contributed to NUE in wheat based on

the physical mapping, sequence analysis and functional validation of an NUE MetaQTL (Quraishi et al., 2011). The role of increasing the efficiency of photosynthesis has also been underlined in favoring the NUtE, and thus grain yield. Under field condition, leaf photosynthetic rate is highly correlated with RuBisCo enzyme content (Hudson et al., 1992). RuBisCo constitutes up to 50% of the total soluble protein content in the leaves of C_3 plants and 20% in C_4 plants (Mae et al., 1983; Sage et al., 1987), which is a major source for N remobilization during senescence (Lawlor, 2002). Thus, the increased content of RuBisCo under high photosynthesis efficiency will provide more N nutrient to the grains from the senescence tissues.

1.6 Use of quantitative trait loci mapping to identify the genomic region of NUE

Quantitative trait loci (QTL) mapping is an efficient method to determine the genomic regions that contribute to NUE under different N environments beginning with Arabidopsis (Rauh et al., 2002; Loudet et al., 2003) and works in more than Arabidopsis. In rice and wheat, numerous QTLs for agronomic traits associated with N use and yield have been mapped (Quarrie et al., 2005; An et al., 2006; Habash et al., 2007; Cormier et al., 2014; Xu et al., 2014; Mickelbart et al., 2015). A glutamatesynthase (*GoGAT*) gene on wheat chromosome 3B contributed to NUE in wheat based on physical mapping, sequence analysis and functional validation of an NUE MetaQTL (Quraishiet al., 2011). In the previous study, the *TaVRN-A1* locus on chromosome 5A was mapped to collocate with a QTL for NUE in wheat, but it was unknown how this locus contributed to NUE in wheat. In this study, we evaluated a population of recombinant inbred lines (RILs) of winter wheat under contrasting N fertilization regimes and identified a major QTL for N-related agronomic traits. We also

cloned the gene associated with this QTL and further validated the gene function related to NUE.

CHAPTER II

GENETIC IDENTIFICATION OF A MAJOR QTL ASSOCIATED WITH NUE

The major results in this study have been published in Plant Biotechnology,* ***Lei Lei*[1]*, Genqiao Li*[1]*, Hailin Zhang, Carol Powers, Tilin Fang, Yihua Chen, Shuwen Wang, Xinkai Zhu, Brett F. Carver, Liuling Yan, 2018. 16 (6): 117-123. ([1]these authors contributed equally to the project). I would like to express my deep thanks to all of the authors for their contributions to the research article.*

Abstract

Increasing NUE in crops is a primary objective for the modern agriculture. Wheat has low NUE (about 33%), but the information about genetic mechanisms controlling NUE is limited. In this study, a major quantitative trait locus (QTL) on chromosome 5A was found associated with NUE (*QNue.osu-5A*) in a winter wheat population of recombinant inbred lines (RILs) derived from the cross of two winter wheat cultivars 'Jagger' and '2174'. Ten important N-related traits were phenotyped, and five of them including heading date, leaf chlorophyll content, grains per spike, grain yield per plant, and harvest index were found associated with *QNue.osu-5A*. Further, this QTL was cloned using a positional cloning method. Seven critical recombinant inbred lines were

tested in both greenhouse and field for the positional cloning of *TaNUE1*. Three candidate genes were found, including *TaVNR-A1*, *TaAGLG1*, and *TaCYB5*. Our findings could serve as the basis for the further study of the candidate genes for *TaNUE1*.

2.1 Introduction

Nitrogen is the most important nutrient for plant development and growth, and soil is often supplemented with N fertilizer to ensure successful seed production and high grain yield for non-N- fixing food crops such as wheat (*Triticum aestivum L.*), rice (*Oryza sativa L.*), and maize (*Zea mays L.*) (Santi et al., 2013). N fertilizer is essential for higher crop yield, but the excess amount of N pollutes the air and water systems (Hirel et al., 2007). Due to the substantial growth of agriculture, NUE has become an important trait for plant research and breeding.

Quantitative trait loci (QTL) mapping is an efficient method to determine the genomic regions for unknown genes that influence quantitative variation of complex traits such as NUE. In rice and wheat, numerous QTLs for agronomic traits associated with N use and yield have been mapped, but each accounts for only a small part (<25%) of the total phenotypic variation (Quarrie et al., 2005; An et al., 2006; Habash et al., 2007; Cormier et al., 2014; Xu et al., 2014; Mickelbart et al., 2015), which limited further molecular cloning of the mapped QTL. There are also challenges to identify candidate genes in QTL mapping studies. For example, the mapping intervals were not narrow enough to contain a reasonable number of genes due to the imprecision of mapping studies (Gallais and Hirel, 2004).

Conventional breeding efforts in the past few decades have been employed to increase

crop grain yield utilizing NUE, and the genetic variations of NUE between varieties have already been observed in different crops including rice, wheat, maize, sorghum, and barley (Kant et al., 2011), however, the genetic mechanism controlling NUE is not clearly known. Wheat has a low NUE, which ranges between 24% and 42% depending upon the N application rate (Han et al., 2015). It is of great interest to identify the gene responsible for NUE in wheat and improve wheat NUE through the genetic mechanisms' study. In the previous study, the *TaVRN-A1* locus on chromosome 5A was mapped to collocate with a QTL for NUE in wheat, but it was unknown how this locus contributed to NUE. In this study, we evaluated a winter wheat recombinant inbred lines (RILs) population under contrasting N fertilization regimes and mapped a major QTL in the *TaVRN-A1* locus for N-related agronomic traits. Then, we positional cloned the gene responsible for NUE in winter wheat using a large backcross population.

2.2 Materials and Methods

Mapping *QNue.osu-5A* in RILs in low-N soil

The population of RILs was generated from a cross between two locally adapted winter wheat cultivars, 'Jagger' and '2174' (Chen et al., 2009). A population of 96 RILs was used to initially map traits related to NUE. A Kirkland silt loam soil was collected from the Cimarron Valley Research Station, OK (USA); the soil was low in N, but had normal levels of other essential nutrients (Table 1). The soil was thoroughly mixed using a cement mixer before being distributed equally into pots. Three plants of a single RIL were grown in each pot (10 cm in diameter and 12 cm in height) with 1.8 kg soil in a greenhouse in Stillwater, OK. The greenhouse was maintained at a constant temperature

of 25/20 °C day/night with long days (16 h light/8 h darkness), sodium lamps were used to provide additional lighting as needed. The winter wheat population was not subjected to vernalization to avoid interactive effects between low temperatures and the traits of interest.

Leaf greenness and total N of aboveground dry biomass were assessed from one of the replicates. Heading date was scored when a single plant had completely emerged from the boot. Leaf chlorophyll content was assayed using a SPAD 502 chlorophyll meter (Konica Minolta Sensing, Inc., Osaka, Japan). The dry weight of the shoot tissues in mature plants was weighed to determine biomass. Harvest index was the ratio of total grain weight to total aboveground biomass. Tiller number was counted from ten plants at the end of the stem elongation stage when tillers reached their maximum number. Spike number was counted at maturity, and the total number of grains from a plant was counted to calculate the number of grains per spike.

Grains were dried at 65 °C for 2 days; grain weight was the weight of the dried grain plus 13% standard moisture per thousand grains (thousand kernel weight). These yield components were phenotyped from three plants tested in the Kirkland soil in the greenhouse. PCR markers for the allelic variation of each gene were developed and mapped in the RILs (Figures 3a-3f). Markers for three candidate genes were developed based on the sequences deposited in GenBank, including JQ915055 for Jagger and JQ915056 for 2174 of *TaVRN-A1*, JQ915057 for Jagger and JQ915058 for 2174 of *TaAGLG1*, JQ915059 for Jagger and JQ915060 for 2174 of *TaCYB5*.

QTL validation and statistical analysis

QNue.osu-5A was identified in the same population of recombinant inbred lines (RILs) grown under two contrasting N fertilization regimes in the same greenhouse. The genetic linkage group utilized to identify genes for stem elongation time (Chen et al., 2009) and vernalization requirement duration (Li et al., 2013) was used to discover *QNue.osu-5A*. WinQTLCart 2.5 (North Carolina State University, Raleigh) (Wang et al., 2007) was used to conduct analyses for the N-related traits using interval mapping (IM). A QTL was declared when the logarithm of the odds (LOD) score exceeded the threshold value of 2.5, and the significance level was at 0.05. LOD values and genetic partitioning of the total phenotypic variation are presented as generated from the QTL program, using standard procedures. One-way analysis of variance (ANOVA) was used to analyze the interactions of the QTLs with N rate for each of the 10 traits using the SAS procedure CORR (SAS Institute, Cary, NC). A QTL was statistically claimed to be regulated by N, when the genotype x nitrogen environment effect was significant.

The mean value of each trait from plants carrying the same allele was used for ANOVA. The t-test was used to determine the significance level to compare mean differences between treatments. Correlation analysis examined the association of two phenotypes in the same treatment.

Testing critical recombinant lines

Fifteen recombinant $BC_1F_{2:3}$ and three recombinant BC_1F_3 lines (Table 3; Figure 2c) were tested with the same Kirkland soil. Sixty plants of each recombinant line were tested under the same greenhouse conditions used to discover *QNue.osu-5A*. A commercial potting mix, Sunshine Redi-earth growing mix (Sungro Horticulture Canada Led.) was

used in the experiments, which includes fine Canadian sphagnum peat moss, vermiculite and dolomitic limestone, and has a nutrient system delivering a supply of N for 6 weeks. Whenever appropriate, N from Miracle-Gro® Water Soluble All Purpose Plant Food or urea fertilizer was dissolved in water and poured into pots. The heading date was scored when a single plant head had completely emerged from the boot. At the Cimarron Valley Research Station in the 2011–2012 growing season, seven critical recombinant lines were grown in a Teller soil with 7.5 ppm NO_3^- N, representing a N-stress condition (Table 1). A forage crop was grown for two consecutive seasons, and no N was supplied to both lower and level the available N in the Teller soil. The experimental design was a randomized complete block with three replications. Yield components were phenotyped from 10 plants per line tested in the Teller soil in the field.

2.3 Results

The discovery of a major QTL for NUE

Two populations from the same 96 RILs and the parental lines ‘Jagger’ and ‘2174’ were initially grown in a greenhouse on Kirkland silt loam soil in which N was severely deficient but other macronutrients were sufficient (Table 1), under long-day conditions and constant temperature, but without vernalization (Figure 1a).

Soil	pH	NO_3-N (ppm)	NH_4-N (ppm)	P (ppm)	K (ppm)	SO_4 (ppm)	Ca (ppm)	Mg (ppm)
Kirkland	5.83	4.38	3.39	55.88	95.50	6.18	466.63	74.62
Teller	6.13	7.50	N/A	28.34	90.50	9.00	786.50	254.67
Soil	**Fe (ppm)**	**Zn (ppm)**	**B (ppm)**	**Cu (ppm)**	**OM (%)**	**Sand (%)**	**Silt (%)**	**Clay (%)**
Kirkland	31.18	1.30	0.12	0.50	0.39	71.70	16.70	11.60
Teller	26.66	0.58	0.21	0.65	0.77	64.60	24.60	10.80

Table 1. Characteristics of the soils used for NUE. Kirkland Silt Loam (Kirkland) that was collected at the Cimarron Valley Research Station Research Station, OK, November 19, 2007. This soil was used in initial experiments in a greenhouse to discover the QTL *QNue.osu-5A*. Teller Loam (Teller) that was located in a field at the Cimarron Valley Research Station in Perkins, OK, October 19, 2012. This soil was used to test seven critical

Under such conditions, the winter wheat plants remained in the vegetative stage and showed similar phenotypes until 11 weeks after planting (Figure 1b). When plants were deprived of N, two different levels of N, 14 mg N kg^{-1} soil (25 N, equivalent to 25 kg N ha^{-1}) and 56 mg N kg^{-1}soil (100 N, equivalent to 100 kg N ha^{-1}), were used to fertilize the plants. Parental lines started to show differences in their morphological traits 3 weeks after N fertilization (Figure 1c).

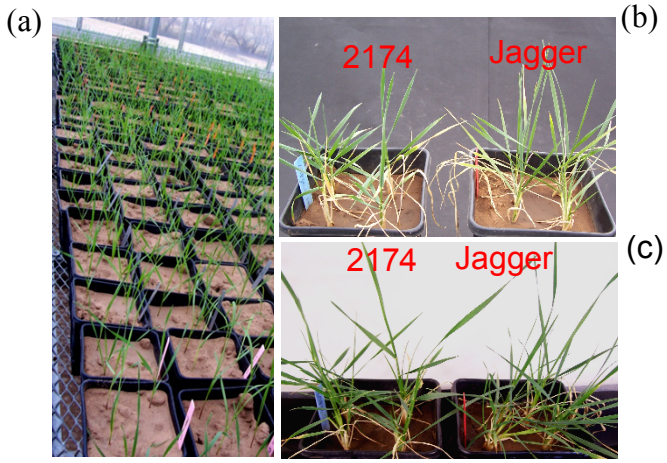
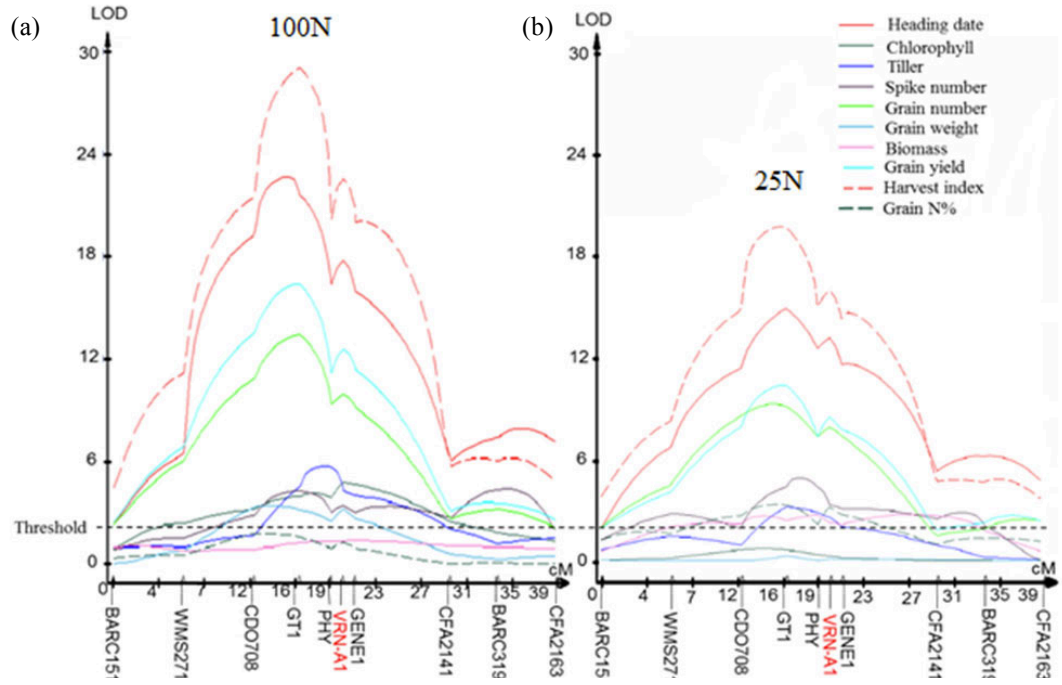


Figure 1. Winter wheat plants in N-deficient soil before and after fertilization. (a) Plants were grown in Kirkland soil that was N deficient and normal for other nutrients. The experiments were conducted in a greenhouse, where temperature, photoperiod and moisture conditions were controlled in order to reduce interactions between genetic and environmental factors on N response. (b) The plants showed similar phenotypes at 11 weeks after planting. (c) The plants showed visible segregation in agronomic and physiological traits three weeks after fertilized with 54.06 mg N kg^{-1} soil (equivalent to 100 kg N ha^{-1}).

As the N-fertilized populations were grown under the same, continuous greenhouse conditions until they reached maturity, 10N-related traits were phenotyped. A major QTL on the long arm of chromosome 5A was mapped associated with the N-related traits, but the colocalized QTLs largely differed in the log of the odds (LOD) for magnitudes of the total variation between the two contrasting N regimes (Figures 2 a, 2b; Table 2).



(c)

Plant ID#	Genetic markers													Kirkland soil						Commercial soil			
	Gene1	PCS		CYB5	AGLG1	VRN1	Pheno		CYS	PHY		GT		STR	BC1F2			BC1F3			BC1F2		
															A	B	H	A	B	H	A	B	H
D1032	H	H	X	B	B	B	B		B	B		B		B	182	203	194				148	147	149
P55	A	A	X	H	H	H	H		H	H		H		H				159	193	-	93	122	-
D695	H	H		H	H	H	H	X	A	A		A		A	174	203	193				92	139	98
T657	H	H		H	H	H	H		H	H	X	B		B	157	191	172	143	212	-	89	159	-
D731	B	B		B	B	B	B		B	B	X	H		H	178	179	175				133	141	142
D1061	A	A		A	A	A	A		A	A	X	H		H	159	163	157				90	94	94
L447	H	H		H	H	H	H		H	H		H	X	A	158	181	161	168	206	-	86	147	-

Figure 2. Genetic mapping and phenotypic effects of *QNue.osu-5A*. Two sets of the Jagger × 2174 recombinant inbred line (RIL) population were evaluated in a temperature- and photoperiod-controlled greenhouse, and in Kirkland soil that was N-deficient but adequate in other essential nutrients for 11 weeks. Then, two different levels of N fertilizer (25N and 100N) were supplied to the plants. (a) QTLs for N-related traits under the 100N condition. The *TaVRN-A1* gene on chromosome 5AL is highlighted in red. (b) QTLs for N-related traits under the 25N condition. The horizontal dashed line represents a threshold value of 2.5 log of the odds (LOD) for N-related traits. (c) Phenotypes (heading dates (days from planting)) and genotypes of seven critical recombinant lines with crossovers within the *QNue.osu-5A* locus. 'A', the Jagger allele; 'B', the 2174 allele; and 'H', the heterozygous state. Pheno represents phenotypes. Three candidate genes are highlighted in red. 'X' indicates a crossover between two neighboring markers.

Traits	100N				25N			
	LOD	R^2 (%)	A	B	LOD	R^2 (%)	A	B
Heading date	22.8	68.2	132	169	14.6	51.0	137	166
Chlorophyll (%)	4.2	19.4	52.2	55.5	NS	NS	40.1	39.1
Tillers per plant	5.8	25.4	6	8.2	3.1	13.9	4.1	5.3
Spikes per plant	4.3	18.6	2.4	3.6	4.8	22.7	1.4	2.4
Grains per spike	13.5	47.4	28.4	13.2	9.1	36.4	14	8.2
Grain weight (TKW)	3.5	15.9	30.1	26.3	NS	NS	28.5	27.6
Grain yield (g/plant)	16.5	55.3	0.84	0.35	10.2	38.5	0.39	0.22
Biomass (g/plant)	1.4	6.4	2.2	2.5	2.6	13.2	1.2	1.4
Harvest index	29.1	74.9	0.385	0.134	19.4	60.9	0.333	0.166
N content (%)	1.8	8.8	1.75	1.92	3.3	15.4	1.89	2.19

Table 2. LOD and variation of *QNue.osu-5A* on ten traits related to N utilization. Ten traits were characterized for two populations grown under different N levels allied to the soils. When the phenotypic traits were analyzed with the genetic map, it was found that a major QTL was associated with variation in all traits studied (Figure 2). The populations were planted on November 18, 2007. Fertilizers were supplied on February 13, 2008. The tiller number was determined on March 11, 2008. Chlorophyll content was measured on March 5, 2008. A: The Jagger allele; B: the 2174 allele.

Furthermore, statistical analyses showed that interactions of the QTLs with N rate were significant or highly significant for five traits including heading date, leaf chlorophyll content, grains per spike, grain yield per plant and harvest index (Table 3). Therefore, these five traits should be regulated by the gene at the QTL.

		DF	ANOVA SS	Mean Square	F Value	Pr > F
Heading date	VRN1	1	43494.343	43494.343	173.06	<0.0001
	N_Rate	1	25.566	25.566	0.1	0.7502
	VRN1*N_Rate	1	1078.415	1078.415	4.29	0.0400
Grains per spike	VRN1	1	4872.42	4872.42	80.11	<0.0001
	N_Rate	1	3734.657	3734.657	61.4	<0.0001
	VRN1*N_Rate	1	899.189	899.189	14.78	0.0002
Grain yield (g/plant)	VRN1	1	4.5222	4.5222	97.42	<0.0001
	N_Rate	1	3.2872	3.2872	70.82	<0.0001
	VRN1*N_Rate	1	1.0709	1.0709	23.07	<0.0001
Harvest index	VRN1	1	17863.089	17863.089	212.88	<0.0001
	N_Rate	1	27.201	27.201	0.32	0.5699
	VRN1*N_Rate	1	831.631	831.631	9.91	0.002
Leaf chlorophyll content (%)	VRN1	1	55.015	55.015	3.91	0.0497
	N_Rate	1	8396.615	8396.615	596.88	<0.0001
	VRN1*N_Rate	1	174.669	174.669	12.42	0.0006

Table 3. Regulatory effects of *QNue.osu-5A* by N. The one-way analysis of variance (ANOVA) was used to determine whether there was any interaction of *TaVRN-A1* representing *QNue.osu-5A* with N rate for N-related traits.

In the greenhouse study, winter wheat plants were not vernalized, to avoid the effect of vernalization; hence, these plants showed large variations in heading date. Those lines carrying the Jagger allele had an earlier average heading date and produced more grains, but lines carrying the 2174 allele showed delayed heading and produced fewer grains. The average grain yield of those lines carrying the Jagger allele was 0.84 g per plant, an increase of 140% compared with 0.35 g per plant for lines carrying the 2174 allele, when plants were grown in the same 100 N soil (Table 2). These results indicated that the N-regulated gene at the QTL affected development and thus grain yield. The QTL on wheat chromosome 5AL for the multiple N-related traits was temporarily designated *QNue.osu-5A*, and the gene causing the QTL was designated *TaNUE1*, the gene shown to have pleiotropic effects in hexaploid wheat.

Positional cloning of the gene associated with *QNue.osu-5A*

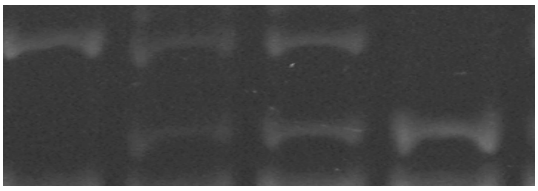
Fifteen recombinant events that occurred at *QNue.osu-5A* were used to fine map the QTL in the Kirkland soil and in the same greenhouse with long days and constant temperatures as used to find *QNue.osu-5A* (Table 4). These recombinant lines were generated from 6410 BC_1F_3 plants that were used to clone the gene responsible for vernalization requirement duration in winter wheat in our previous study (Li et al., 2013).

Plant ID#	V1G1	PCS		CYB5	AGLG1	VRN1	Pheno		CYS	PHY		GT		STR	KIN		CBP		USPC3	MET		EX1	AMT	CDO708	BC1F2:3			BC1F3		
																									A	B	H	A	B	H
D1032*	H	H	X	B	B	B	B		B	B		B		B	B		B		B	B		B	B	B	182	203	194			
P55*	A	A	X	H	H	H	H		H	H		H		H	H		H		H	H		H	H	H				*159*	*193*	-
D695*	H	H		H	H	H	H	X	A	A		A		A	A		A		A	A		A	A	A	*174*	*203*	*193*			
T657*	H	H		H	H	H	H		H	H	X	B		B	B		B		B	B		B	B	B	*157*	*191*	*172*	*143*	*212*	-
B562	H	H		H	H	H	H		H	H	X	B		B	B		B		B	B		B	B	B	*144*	*170*	*152*			
D594	B	B		B	B	B	B		B	B	X	H		H	H		H		H	H		H	H	H	187	199	200			
D731*	B	B		B	B	B	B		B	B	X	H		H	H		H		H	H		H	H	H	178	179	175			
D1061*	A	A		A	A	A	A		A	A	X	H		H	H		H		H	H		H	H	H	159	163	157			
L447*	H	H		H	H	H	H		H	H		H	X	A	A		A		A	A		A	A	A	*158*	*181*	*161*	*168*	*206*	-
A210	A	A		A	A	A	A		A	A		A		A	A	X	H		H	H		H	H	H	108	124	113			
T485	A	A		A	A	A	A		A	A		A		A	A	X	H		H	H		H	H	H	160	157	167			
T1013	A	A		A	A	A	A		A	A		A		A	A	X	H		H	H		H	H	H	160	163	160			
CAP7	B	B		B	B	B	B		B	B		B		B	B		B	X	A	A		A	A	A		189				
T722	A	A		A	A	A	A		A	A		A		A	A		A	X	H	H		H	H	H	133	138	140			
CAP6	A	A		A	A	A	A		A	A		A		A	A		A		A	A	X	B	B	B	145					
Jagger	A	A		A	A	A	A		A	A		A		A	A		A		A	A	A	A	A	A	155					
2174	B	B		B	B	B	B		B	B		B		B	B		B		B	B	B	B	B	B		171				

Table 4. Wheat heading date of seven critical recombinant lines and parental lines tested in different soils. **** Markers flanking *QNue.osu-5A* were developed in our previous study (Li, et al. 2013). '*' indicates those lines were tested in both Kirkland and commercial soils. Markers for six genes used to narrow down the *TaNUE1* region include *GT*, *STR*, *KIN*, *CBP*, *USPC3*, and *EX1*.

PCR markers for the allelic variation of each gene were developed and mapped in the RILs (Figures 3a-3f). Seven of these recombinant lines were selected for testing in the commercial soil. Based on the segregation of heading date in these recombinant lines (Figure 2c; Table 4), the location of *TaNUE1* was narrowed down to a region containing three candidate genes, *TaCYB5*, *TaAGLG1*, and *TaVRN-A1* (Figure 2c).

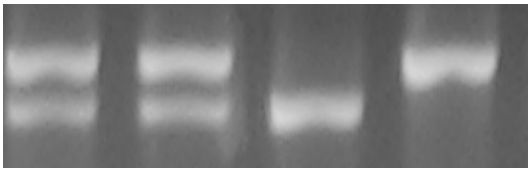
(a) **Marker for *GT***



Jagger Het. Het. 2174

Primers for mapping of the *GT* gene are GT-F6 (5'-CAGGTACGTGACAGAGATCGA-3') and GSGT-R3 (5'-CCTCTGCCAATCCAGACGATGG-3'). The primers amplified 789 bp fragment by using regular PCR at 55ºC for annealing temperature and 1 min for extension. The PCR products were digested with restriction enzyme *Rsa* I, and he polymorphic bands were 257 bp for the Jagger allele and 224 bp for the 2174 allele.

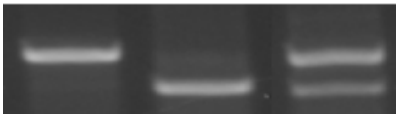
(b) **Marker for *STR***



Het. Het. Jagger 2174

Primers for the *STR* gene are STR-C2F (5'-ATCGTGTAGAGGTGCAGCAACTA-3'and STR-R3 (5'-TGAAAGATCCCAGGGACAAACTA-3'). The primers amplified ~890 bp fragment (PCR sequence was sent to sequence directly, so no exact size was available) by using regular PCR at 55ºC for annealing temperature and 1 min for extension. The PCR products were digested with restriction enzyme *Hae* III, and the polymorphic bands were 480 and 410 bp for the Jagger allele and ~890 bp for the 2174 allele

(c) **Marker for *KIN***

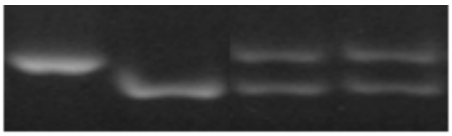


491 bp
342 bp

Jagger 2174 Het.

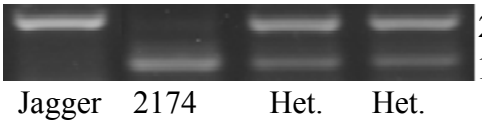
Primers KIN5A3endF4 (5'-CTTGACTATTTTGTTGTTTATCATAATC TGTGG-3') KIN3endR4 (5'-GAAAACAACGACCAAATAGGCGAGC-3') were used to map *KIN*. These primers amplified 710bp from Jagger and 722bp from 2174 annealing temperature 60℃ extension time 45sec. This marker was polymorphic between Jagger and 2174 with restriction enzyme *Rsa* I.

(d) **Marker for *CBP***



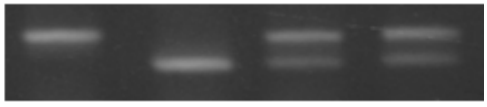
Primers CBP_PromF3 (5'-CTTTATGGCATGAAAATTTTGATGTCATTG-3') CBP_Prom_C6R2 (5'-ACTGTATAGACTCGAAGAGAGTGCA-3') were used to map the *CBP* gene. These primers amplified 176 bp fragment from Jagger and 167 bp fragment from 2174 using annealing temperature 60℃ and extension time 40sec. The PCR products are polymorphic between Jagger and 2174.

(e) **Marker for *USPC3***



Primers USP1-C3-MF1 (5'-AAGCCACGCCTTGACCCTGTG-3') and USP1-C3-MR1 (5'-AAAGAACACTACGTCCATGGCAATGTAATG-3') were used to map the *USPC3* gene. These primers amplified 348bp fragment annealing temperature 60℃ extension time 50 sec. This marker was polymorphic between Jagger and 2174 with restriction enzyme *PhyCH4* III.

(f) **Marker for *EX1***



Primers EX1F4 (5'-GCTTCTCCTGGAAGCTGCCGC-3') and EX1C3R3 (5'-TGTCACTAAATCTCGTTAAATCCCATCATTG-3') were used to map the *EX1* gene. These primers amplified 422bp fragment annealing temperature 60℃ extension time 50sec.This marker was polymorphic between Jagger and 2174 with restriction enzyme *Nhe* I.

Figure 3. Marker for genes encompassing *TaNUE1.*

The seven recombinant lines were also tested in a field with Teller loam soil with 7.5 ppm NO_3^- N, representing an N-stress condition (Table 1). As shown in Figure 4, plants carrying the Jagger *TaNUE1* allele showed an increase of 18.1% in grain yield (Figure 4a), due to a significant increase in grains/spike (Figure 4b) and biomass (Figure 4c), compared with plants carrying the 2174 *TaNUE1* allele in the low-N scenario. These field

results further confirmed that *TaNUE1* in the targeted region affected grain yield in the low-N scenario.

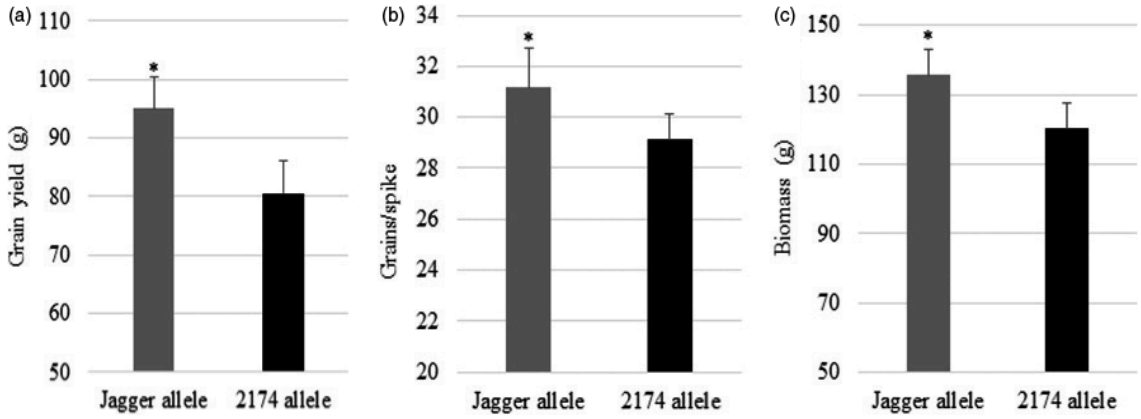


Figure 4. Phenotypic effects of *TaNUE1* in the field. Several critical recombinant lines were tested in a field at Oklahoma State University Cimarron Valley Research Station in the 2011–2012 growing season. (a) Grain yield. (b) Grains per spike. (c) Biomass. The average values of each genotype in each critical recombinant line were compared, and the bars indicate standard errors. n = 6 for the Jagger allele, and n = 8 for the 2174 allele. Asterisk indicates that the difference was significant between the two alleles ($Pr < 0.05$).

2.4 Discussion

In this study, we characterized a QTL related to important N related traits and linked to *TaVRN-A1* locus. Ten N-related traits were phenotyped and five of them including heading date, leaf chlorophyll content, grains per spike, grain yield per plant and harvest index showed significant or highly significant in the interactions between the QTLs and N rates. These findings indicated that these five traits were regulated by the gene representing the QTL.

The two populations along with parental lines of this study were grown under N-deficient soil and then applied two contrasting rates of N fertilizer. There was no significant

difference observed on the morphological traits during young seedling stage indicating that these two cultivars have similar tolerance to N stress. After fertilization with 100N and 25N respectively, these population of plants showed significant segregation in agronomic and physiological traits.

When using two contrasting N fertilization regimes to map *QNue.osu-5A*, we had the following findings: *QNue.osu.5A* was associated with a strong response to N fertilizer. Five of ten phenotypes characterized were regulated by N.

In cereal crops, NUE refers to the ratio of grain yield to N supplied by soil and fertilizer, which is dissected into two components: N uptake (NU) and N utilization efficiency (UTE) (An et al., 2006; Moll et al., 1982). In this study, we used grain yield to describe NUE for the two alleles, which were tested at the same N level. The allelic effect of Jagger allele on grain yield was increased to up to 140% compared with 2174 allele when the population was grown in the Kirkland soil with limited N in the greenhouse without vernalization. This result indicates that the Jagger allele has higher NUE than 2174 allele.

As described in previous studies, the *TaVRN-A1* locus on chromosome 5AL was also mapped and directly linked to key wheat and barley traits such as frost tolerance, spike architecture and plant height, tiller and spikelet number, leaf length, grain yield, and nitrogen uptake and NUE (An et al., 2006; Deng et al., 2015; Fowler and Gusta, 1977; Habash et al., 2007; Kato et al., 2000; Quraishi et al., 2011; Roberts, 1990; Snape et al., 1985). In addition to the *Vrn-A1* locus, the *Vrn-D1* locus was associated with a QTL for traits related to NUE components in wheat in the same population of the Chinese Spring 9 × SQ1 population (Habash et al., 2007). However, the genes in the mapped regions that

controlled NUE and the related agronomic traits were unknown. Our positional cloning study revealed three candidate genes for *TaNUE1* including *TaVRN-A1*, *TaAGLG1*, and *TaCYB5*. This study provided the basis for validating of the candidate genes for *TaNUE1*.

CHAPTER III

VALIDATION OF CANDIDATE GENES FOR *TaNUE1*

The major results in this study have been published in Plant Biotechnology,* ***Lei Lei*[1]*, Genqiao Li*[1]*, Hailin Zhang, Carol Powers, Tilin Fang, Yihua Chen, Shuwen Wang, Xinkai Zhu, Brett F. Carver, Liuling Yan, 2018. 16 (6): 117-123. ([1]these authors contributed equally to the project). I would like to express my deep thanks to all of the authors for their contributions to the research article.*

Abstract

In our previous study, we cloned a major quantitative trait locus *QNue.osu-5A* for N-related agronomic traits and identified three genes as candidates for *TaNUE1*. In this study, we found that the vernalization gene *TaVRN-A1* was the *TaNUE1* gene. Due to the Ala^{180}/Val^{180} substitution, *Ta*VRN-A1a protein encoded by the Jagger allele and *Ta*VRN-A1b encoded by the 2174 allele had differential interactions with *Ta*ANR1 protein, which is encoded by a wheat orthologue of *Arabidopsis nitrate regulated 1* (ANR1). A natural mutant of *TaANR1* was found resulting in missing exon 6 in its mRNA, which had genetic effect on wheat development and growth. The transcripts of both *TaVRN-A1* and *TaANR1* were down-regulated by N. The *Ta*VRN1 protein was found to regulate sugar content in leaves by its direct biding to the CArG

promoter of *Ta6SFT1* for sucrose:fructan 6-fructosyltransferase in C metabolism. The unraveling of interactions of *Ta*VRN-A1 protein with the wheat proteins and DNA elements has laid the foundation for us to understand NUE and enter the gene/protein networks for NUE in wheat.

3.1 Introduction

Nitrogen is one of the most important nutrients to crop growth and development. Applying N fertilizer to crops is an effective way to increase yield. However, only 30–35% of the added N fertilizers are taken up and used by wheat plants in the year of application, and the remaining 65–70% (assuming fertilizer–soil equilibrium) is lost (Gaju et al., 2011; Raun and Johnson, 1999). Developing wheat varieties that require less N input yet maintain the same or higher grain yield is an economically and environmentally sustainable goal in international agriculture. In the previous study, three candidate genes were cloned for a major QTL associated with NUE.

Candidate genes of *TaAGLG1* and *TaVRN-A1* are both from MADS (MCM1/AGAMOUS/DEFICIENS/SRFI)-box gene family. MADS-box genes encode transcription factors, which are known to be involved predominantly in several plant developmental processes or signal transduction processes. All MADS-box genes encode proteins containing a DNA-binding domain characterized by a highly conserved 180 bp DNA sequence in MADS-box gene that binds a consensus recognition DNA sequence known as CArG boxes [$CC(A/T)_6GG$] (Parenicova, 2003; Riechmann et al., 1996; Hayes et al., 1998). The MIKC-type MADS-box proteins have been widely studied because of their roles as transcriptional regulators which were composed of different domains for efficient DNA-binding, transcriptional activation or repression, and protein-protein

interactions (Theissen et al., 1996). The candidate *TaVRN-A1* was one of the best-studied plant MADS-box family gene which functions in determining floral organ identity and the flowering time regulation. *VRN1* encodes a MADS-box transcription factor, which is the wheat orthologue of the Arabidopsis meristem identity gene *APETALA1* (*AP1*). *VRN1* was cloned for qualitative vernalization requirement between spring wheat and winter wheat (Yan et al., 2003). In both wheat and barley, the plants carrying the wild-type *VRN1* allele showed winter growth habit. Natural mutations in the promoter or first intron of the wild-type allele produced dominant *Vrn1* alleles that showed spring growth habit (Yan, et al., 2004; Fu, et al., 2005; von Zitewitz, et al., 2005; Cockram, et al., 2007). *VRN1* was required for the transition from the vegetative phase to the reproductive phase that was upregulated by *VRN3,* which was a promoter of flowering integrating signals from the photoperiod and vernalization (Yan et al., 2006; Trevaskis et al., 2007). However, *VRN3* was repressed by *VRN2*, a repressor of flowering under long days, before vernalization (Takahashi and Yasuda, 1971; Laurie, et al., 1995; Dubcovsky, et al., 1998; Distelfeld, et al., 2009). *VRN1* was also cloned for quantitative vernalization requirement between weak winter wheat and strong winter wheat (Li et al., 2013). Due to the Ala^{180}/Val^{180} mutation in the protein, proteins from two winter wheat alleles have the differential binding ability with *Ta*HOX1 (the first homeobox protein in *Triticum aestivum*), which has been found to be upregulated by low temperature and have the genetic effect on heading date.

Another candidate gene *AGLG1* is the wheat orthologue of Arabidopsis meristem identity gene *AGL2*, which may be involved in spike differentiation (Yan et al., 2003). The function of *AGL2* in Arabidopsis may play a fundamental role in the development of all

floral organs, as well as seeds and embryos (Flanagan and Ma, 1994).

The functions of MIKC-type MADS-box genes are not restricted to the regulation of the transition to flowering and development of reproductive structures (Rijpkema et al., 2007), these are expressed throughout the plants and some of them are root specific. It has been reported that at least 50 MADS-box genes are expressed in Arabidopsis roots, however, function of most of these genes are not known (Gan et al., 2005). Several MADS-box genes such as *ANR1*, *AGL14*, *AGL16*, *AGL19*, *SOC1*, *AGL21*, *AGL26* and *AGL56* (*NRT1.1*) have been reported to respond to N supply, and *ANR1*, *AGL19*, *AGL26* and *AGL56* (*NRT1.1*) are identified as root specific expression (Gan et al., 2005). *ANR1* was the first identified gene from MADS-box gene family with known function as a key regulator of lateral root growth in response to signals from external NO_3^- in Arabidopsis (Zhang and Forde, 1998). Over expression of *ANR1* in Arabidopsis showed pleiotropic effects including rapid early seedling development, increased length and number of lateral roots, and increased shoot fresh weight (Gan et al., 2012). *ANR1*-dependent signaling pathway was activated by NRT1.1, a nitrate transporter, which facilitated the uptake of auxin in response to NO_3^- and affected the root branching (Krouk et al., 2010). There was a considerable potential that *ANR1* was post-translationally regulated by external nitrate-dependent signals, either through direct modification or through interactions with other proteins because that the MADS-box proteins were known to function as homodimeric or heterodimeric complexes interacting with proteins of other kinds (Immink et al., 2010).

K-domain from MADS-box proteins mediates protein interaction between MIKC-type MADS proteins. The K-domain may lead to higher-order protein complex formation,

which enables ternary interaction among homologous proteins with the same MIKC-type MADS-box protein family. It significantly increases the number of interacting transcriptional factors compared with dimeric interaction (Kaufmann et al., 2005). The C-terminal domain enhances or stabilizes the complex during interactions, which is mediated by the K-domain (Fan et al., 1997; Pelaz et al., 2001). With these characters for different domains, MIKC-type MADS-box proteins play an essential role in plant development as a transcription factor. Both *Ta*VRN1 and *Ta*AGLG1 are from MICK-type MADS box protein family, thus, in this study, we try to find interactors of these MADS-box proteins from cloned candidate genes and then study its effect on processes in N regulation pathway and plant development.

Nitrogen is required by plants to synthesize protein that contributes to the crop biomass accumulation along with carbohydrates (Sulpice et al., 2009). This is maintained by complex metabolic machinery in C/N networks regulating many essential processes, for instance the uptake and assimilation of N, allocation of N between different organs, and many aspects of plant growth and development including shoot: root ratio, root architecture, and flowering (Nunes-Nesi et al., 2010). Fructan are soluble polymers of fructose and an essential temporary storage carbohydrate in wheat. Wheat fructans, like barley and oat, consist of a mixture of graminans, i.e. linear and branched fructans containing both β-(2,1) and β-(2,6) fructosyl linkages (Kawakami and Yoshida, 2005). Sucrose:fructan 6-fructosyltransferase (*6SFT1*) is necessary for the synthesis of branched-type fructans such as graminans with β-(2,6) fructosyl linkages (Kawakami and Yoshida, 2005). Expression level of fructosyltransferase genes is directly correlated with fructosyltrasferase activity, thus, higher transcript level promotes fructan

accumulation. In a previous study, under N deficient conditions, wheat stem showed high fructan synthesis rate, which correlated with the upregulation of *6SFT1* (Ruuska et al., 2007). In winter wheat, temporarily stored fructans protect plants from various environmental stresses including N deficiency, drought, and low temperature (Kawakami and Yoshida, 2005; Ruuska et al., 2007), and later contribute singnificantly to grain yield under stressed conditions (Mcgrath et al., 1997). In this study, a CArG box, a consensus recognition DNA sequence by MADS-box protein, was found in the promoter of *Ta6SFT1*. We will identify if the protein for the cloned gene has interaction with *Ta6SFT1* and then test if it involves in the regulation of sugar synthesis.

3.2 Materials and Methods

In vitro protein interactions

The three cDNAs of *TaVRN-A1a* and *TaVRN-A1b* encoding proteins from 85–191 a.a., 85–179 a.a., and 139–191 a.a.; one cDNA of *AGLG1a* and *AGLG1b* encoding proteins from 1 to 180 a.a., were each cloned into a pSKB3 vector with an N-terminal 6×HIS-tag (Li et al., 2013). The cDNAs encoding proteins from 111 to 240 a.a.(end) for *Ta*ANR1a, and from 111 to the end for *Ta*ANR1b, were cloned into a pMAL-c2X vector with an MBP-tag (New England BioLabs, Ipswich, MA, USA). The primers used for cloning are listed in Table 7. Constructs were expressed in E. coli (BL21 DE3). A Ni-NTA column (Qiagen, Germantown, MD, USA) was used to purify the expressed proteins from pSKB3 with the 6×HIS-tag, and an amylose column (New England BioLabs) was used to dialyse and purify the protein fused with the MBP-tag. The protein fused with the 6×HIS-tag (500 μg/mL, 50 μL) was incubated in an Ni-NTA column (40 μL) for 1 h at 4 °C in

binding buffer with 10 mM imidazole, and the MBP-tag protein (500 μg/mL, 100 μL) was then added to the column. MBP-tag protein (500 μg/mL, 100 μL) alone was added to a Ni-NTA column as a negative control for nonspecific interactions between MBP-tag and the Ni-NTA column. Reactions were incubated overnight at 4 °C. The interacting proteins in the resin were then washed with chilled binding buffer with 35 mM imidazole six or eight times until the MBP-tag protein disappeared in the negative control. The protein was eluted by boiling the samples in 40 μL of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer for 5 min. The eluted protein samples (5 μL) were analyzed on a 12% SDS-PAGE gel stained with Coomassie Brilliant Blue. When the protein fused with the MBP-tag was incubated in the amylose column, the 6×HIS-tag protein was added to the amylose column and then washed with column buffer until the 6×HIS-tag protein disappeared in the negative control. Interacting proteins were analyzed in pull-down assays. ImageJ 1.32 software (National Institutions of Health, Bethesda, MD. http://rsb.info.nih.gov/ij) was used to quantify intensity of interacting proteins. The amount of interacting proteins was converted to the percentage of its own intensity over the proteins collected from the same reaction.

Subcellular localization and *in vivo* protein interactions

The complete cDNA of *TaANR1a* encoding its protein from 1 to 150 a.a. was cloned into pDONR207 with the BP Cloning Kit (Invitrogen) according to the manufacturer's instructions. The LR Cloning Kit (Invitrogen,Waltham, MA, US) was then used to transfer *TaANR1* to pEarleygate 101 (pEG101) for subcellular localization of *Ta*ANR1a. Primers used for constructs are provided in Table 7. Previous reports located *Ta*VRN-A1 in the nucleus of the cell (Li et al., 2013). The *TaANR1* in pDONR207 was fused to the

C-terminal amino acid portion (175–239 a.a.) of YFP in the pEarleyGate202-YC vector (pEG202-YC) to test *in vivo* interactions with *Ta*VRN-A1 (1–244 a.a, end), which was fused to the N-terminal 174 amino acid portion (1–174 a.a.) of YFP in the pEarleyGate201-YN vector (pEG201-YN). Reciprocal empty vectors were also used as negative controls for interactions with *Ta*ANR1 and *Ta*VRN-A1 proteins. *Agrobacterium tumefaciens* strains (GV3101) carrying the BiFC constructs and p19 strain were used together to infiltrate *Nicotiana benthamiana* leaves 5 weeks after planting. Leaves were ready for BiFC imaging after 3 days of infiltration. Images were visualized under a BX-51 fluorescence microscope (Olympus). Images were taken with a bright filter (BF) to indicate the background of the leaves infiltrated with *A. tumefaciens* carrying constructs, or with an ultraviolet filter to indicate the position of the nucleus stained with 4', 6-diamidino-2-phenylindole (DAPI), and with GFP and GFPA filters to indicate the presence of fluorescent proteins. Scale bars in all images are 50 μm.

Transgenic wheat

Two transgenic plants (T20 and T40) were generated using the *TaVRN-A1::RNAi* constructs, and three transgenic plants (T1, T13 and T14) were generated using the *TaANR1::RNAi* construct. The primers used to amplify the genes are provided in Table 7. Amplified genes were inserted at the enzyme digestion sites (Asc I/Avr II, sense fragment; Spe I/Asis I, antisense fragment) of the RNAi vector pMCG161—this contains the *BAR* gene, which confers resistance to bialaphos. Constructs were transformed into the Jagger cultivar by particle bombardment using a published protocol (Sparks and Jones, 2014). Transgenic wheat plants were tested in the same Kirkland soil used to discover *QNue-osu-5A*. The T_1 of three lines of *TaANR1:: RNAi* were tested in the

commercial soil. All transgenic lines were tested under the same greenhouse conditions used to discover *QNue-osu.5A* and without vernalization. Leaf samples were collected from *TaVRN-A1::RNAi* lines 77 days after planting and before N fertilizer at the 100 kg N ha^{-1} level was applied to positive and negative plants. Leaf samples with/without N were collected for RNA extraction 3 weeks after fertilization. Root samples of *TaANR1::RNAi* lines were collected 56 days after planting and before N fertilizer at the 100 kg N ha^{-1} level was applied. Root samples for RNA extraction were collected 1 and 2 weeks after fertilization. Four T_1 lines of T20 and T40 were selected to generate a T_2 population for phenotyping in the commercial soil.

Quantitative RT-PCR

Total RNA from shoots and roots was extracted using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. Root RNA was extracted using a modified method in the RNA precipitation step, whereby 0.25 mL of isopropanol was added, followed by 0.25 mL high salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl) per 1 mL TRIzol® reagent. One microgram of RNA was treated with deoxyribonuclease I (Invitrogen), and complementary DNA (cDNA) was synthesized using a SuperScriptTM II Reverse Transcriptase Kit (Invitrogen) with an oligo$(dT)_{20}$ primer. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was carried out using a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) and iQTM SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) with a three-step cycling program consisting of an initial denaturation step at 95 °C for 3 min, followed by 39 cycles at 95 °C for 15 s, 57 °C for 30 s and 72 °C for 30 s. Primers used in RT-qPCR to amplify *TaVRN-A1*, *TaANR1* and actin are listed in Table 6. Gene

transcript levels were described using the values calculated by the $2^{(-\Delta\Delta CT)}$ method (Li et al., 2013), where CT is the threshold cycle. All samples were subjected to two technical replicates and at least six biological replicates.

Regulation of *TaVRN-A1* by N in different cultivars/lines

Three cultivars/lines carrying the *TaVRN-A1a* allele (Bentley, OK12716R/W, and OK11D25056) and four cultivars carrying the *TaVRN-A1b* allele (Duster, Gallagher, IBA, and Ruby Lee) were first planted in the field for an annual variety trial at Stillwater on 14 October 2015. Plots were 3.0 m in length, with 30 cm between rows. At the experiment site, the top 30 cm of soil contained 47 ppm N, 39 ppm P_2O_5 and 66 ppm K_2O, with a pH of 5.3. Diammonium phosphate (DAP, 18-46-0) fertilizer of 56 kg/ha was also supplied in-furrow. On 24 February 2016, 130 days after planting, six plants of each cultivar, which were expected to have satisfied their vernalization requirement in the natural field condition, were transferred to pots with commercial soil in the greenhouse and N fertilizer at the 100 kg N ha^{-1} level was supplied. Three weeks after fertilization, leaf samples from three plants were collected to test the regulation of gene expression by N. Unfertilized plants were used as controls.

Electrophoretic mobility shift assay (EMSA)

The *Ta6SFT1* promoter fragment containing the CArG-box was amplified from Jagger using Phusion® High-Fidelity DNA Polymerase (New England BioLabs) with primers indicated as underlined sequences in Figure 12a to amplify the 150-bp promoter region of *Ta6SFT1*. The promoter region prior to the transcriptional site is determined based on the comparison of the *Ta6SFT1* gDNA sequence (TGACv1_scaffold_288169_4AL) and

cDNA sequence of CA501985. Pierce™ Biotin 3' End DNA Labeling kit (Thermo Fisher) was used for labeling the 3'-OH end of the double-stranded DNA according to the manufacturer's instructions. Purified proteins *Ta*VRN1a (1-180 a.a.) MBP-tag, *Ta*VRN1b (1-180 a.a.) MBP-tag, and *Ta*ANR1 (1-180 a.a.) MBP-tag were used in EMSA. EMSA was performed with the Light Shift® Chemiluminescent EMSA Kit (ThermoFisher Scientific). The binding reaction was performed in the mixture, which included 10 mM TRIS (pH 7.5), 50 mM KCl, 1 mM DTT, 2.5% glycerol, 0.05% NP-40, 5 mM $MgCl_2$, 0.5 mM EDTA, 5 μg μl^{-1} poly (dI.dC), 1μg recombinant fusion protein, and 100 fmol biotin-labeled DNA (Zhang et al., 2010). The reaction mix was kept at room temperature for 40 min before loading buffer was added. Total volume of 20 μl of the reaction mix was loaded into 6% native polyacrypamide gel and electrophored. After blotting on a positively charged nylon membrane (Amersham), the DNA was linked using a UV-light crosslinker (VWR) equipped with 254 nm bulbs at 120 mJ cm^{-2} for 45 sec. The membrane was washed with buffers provided by the kit and exposed in FluorChem System (Protein Simple) for 1.5-5 sec according to the manufacturer's instructions.

Water-soluble carbohydrate analysis

Water-soluble carbohydrates (WSC) were extracted from 5-10 mg dried, ground leaf materials using 0.5-1 ml of 80% (v/v) ethanol in H_2O at 80°C, followed by extraction twice using the same volume of H_2O at 60°C to incubate for 60 min and centrifuged at 3,400 rpm at room temperature for 10 min (Ruuska et al., 2007). The combined solutions of three extractions were enzymatically measured for sucrose, glucose and fructose content spectrophotometrically using a Sucrose/Fructose/D-Glucose Assay Kit (Megazyme) according to the manufacturer's instructions. A standard 96-well microtiter

plate with 200 µl per well was assayed in a Synergy™ H1 Multi-mode microplate reader (BioTek), and the content of a sugar was determined by comparing read value to a standard curve that was established using a series of known concentrations of sucrose, glucose or fructose. Three replicates were conducted for each sample.

During the 80% (v/v) ethanol extraction, fructans were not solubilized and remained in the residue. The residue was suspended in 0.5-1 ml of 0.2 M KOH and placed in a boiling water bath for 30 min to break down cell-wall material. After cooling, 5-10 µl of concentrated acetic acid was added to neutralize the solution, and the suspension was used to determine fructan content. 100 µl of the suspension was mixed with 100 µl of 1.2 M perchloric acid and incubated at 60°C for 1 h to hydrolyze fructan. The mixture was neutralized with 60 µl of 2 M K_2CO_3, and centrifuged at 7,000 rpm at room temperature for 2 min. The amount of released fructans in the supernatant was measured using the protocol as previously described (Jenkins et al., 2002).

3.3 Results

Identification of candidate genes for *TaNUE1*

Among the three candidate genes, only *TaVRN-A1* showed allelic variation in the promoter and introns, but there was no significant difference in gene expression between the two *TaVRN-A1* alleles before N was used and within 2 weeks after N was used (Figure 5.). The result suggested that the effects of *TaNUE1* on the mapped traits should rely on its protein sequence but not the transcript level.

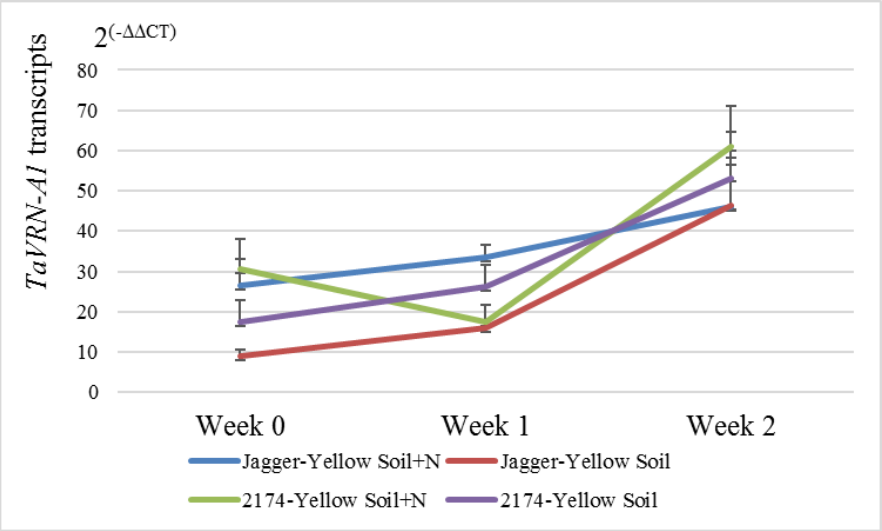


Figure 5. Comparison of *TaVRN-A1* expression level in Jagger versus 2174. The plants were grown in the same Kirkland soil which was N-deficient with normal levels of other nutrients used for the discovery of *QNue.osu-5A* and in a greenhouse, where temperature, photoperiod and moisture conditions were controlled in order to reduce interactions between genetic and environmental factors on N response. The plants were stressed for 11 weeks from planting by low soil N. The two leaf samples were collected for gene expression analysis before N was used (Week 0), and one week (Week 1) and two weeks (Week 2) after N was used. *TaVRN-A1* alleles, *TaVRN-A1a* for Jagger and *TaVRN-A1b* for 2174, did not show significant difference in transcript level. RT-PCR was used to determine transcriptional levels of leaf RNA samples by the SYBR Green PCR Master Mix, and actin was used as an endogenous control. Gene transcriptional levels are described using values calculated by the $2^{(-_{\Delta\Delta}CT)}$ method, where CT is the threshold cycle. The values represent mean expression levels (n=21, t=0.687), and the error bars indicate standard errors.

Among the three candidate proteins for *Ta*NUE1, there were no differences between the proteins encoded by the two alleles of *TaCYB5*; this gene was thus excluded as a candidate. At the protein sequence, *Ta*AGLG1 had one amino acid substitution, whereas *Ta*VRN-A1 had two. Next, we tested whether the two MADS proteins, *Ta*AGLG1 and *Ta*VRN-A1, interacted with the wheat orthologue of *ANR1* in Arabidopsis, which is also a MADS-box protein. *ANR1* has a key role in regulating lateral root growth in response to changes in the external NO_3^- supply (Zhang and Forde, 1998). *TaANR1* (Genbank

Accession Number AM502900) had 70% similarity to the Arabidopsis *ANR1* (GeneBank Accession Number CAB09793). MADS-box proteins may form a protein complex, in which one MADS-box protein may positively or negatively regulate the expression of another MADS-box gene via a direct interaction (Jack, 2004). *TaVRN-A1*, *TaAGLG1* and *TaANR1a* from Jagger were expressed in Escherichia coli. In pull-down assays, no interaction was observed between *Ta*ANR1a and *Ta*AGLG1 (Figure 6a). However, the two *Ta*VRN-A1 proteins encoded by the Jagger and 2174 alleles interacted differently with the *Ta*ANR1a protein (Figures 6b–6e).

The alignment of *Ta*VRN-A1 and *Ta*ANR1a proteins suggested that they might have interacting sites in the K box (Figure 7). The critical sites at which the Jagger *Ta*VRN-A1a and 2174 *Ta*VRNA1b proteins interact with *Ta*ANR1a were identified by three experiments. *Ta*VRN-A1a(85–191) and *Ta*VRN-A1b(85–191), which included both Leu^{117}/Phe^{117} and Ala^{180}/Val^{180} substitutions, interacted differentially with *Ta*ANR1a (Figure 6b). Similarly, *Ta*VRN-A1a(131–191) and *Ta*VRN-A1b(139–191), which included an Ala^{180}/Val^{180} substitution but not a Leu^{117}/Phe^{117} substitution, interacted differentially with *Ta*ANR1a (Figure 6c). However, *Ta*VRN-A1a(85–179) and *Ta*VRN-A1b(85–179), which included a Leu^{117}/Phe^{117} substitution but not an Ala^{180}/Val^{180} substitution, interacted with *Ta*ANR1a in a similar manner (Figure 6d). The intensity of interacted *Ta*ANR1a proteins was significantly stronger in the pull-down *Ta*VRN-A1b protein than the pull-down *Ta*VRN-A1a protein including the Ala^{180}/Val^{180} substitution (Figure 6e). These comparative in vitro interaction studies indicated that the differential interaction of *Ta*VRN-A1a and *Ta*VRN-A1b with *Ta*ANR1a was caused not by the Leu^{117}/Phe^{117} substitution but by the Ala^{180}/Val^{180} substitution.

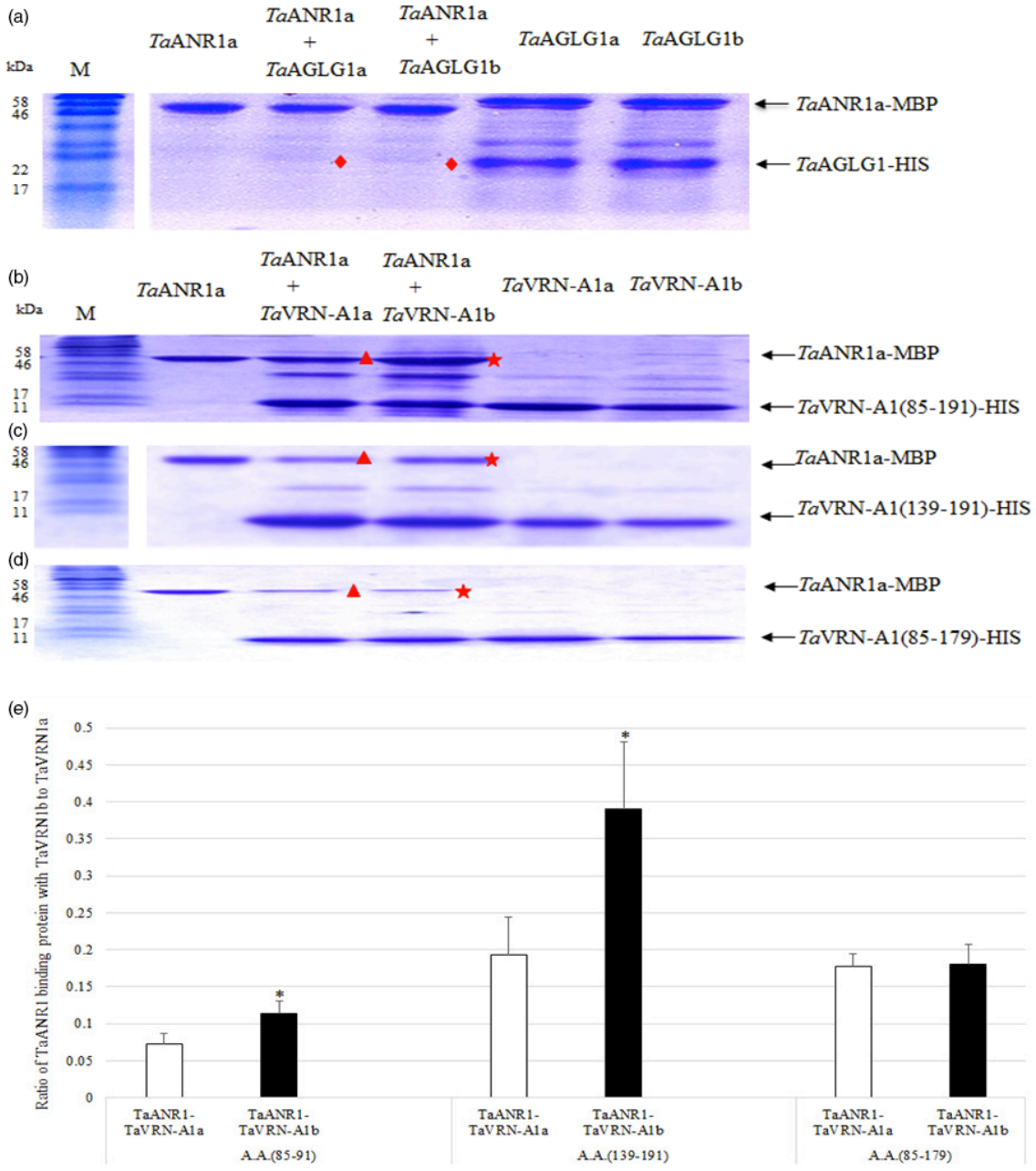

(a)
kDa
M
TaANR1a
TaANR1a + TaAGLG1a
TaANR1a + TaAGLG1b
TaAGLG1a
TaAGLG1b
58
46
22
17
TaANR1a-MBP
TaAGLG1-HIS
(b)
kDa
M
TaANR1a
TaANR1a + TaVRN-A1a
TaANR1a + TaVRN-A1b
TaVRN-A1a
TaVRN-A1b
58
46
17
11
TaANR1a-MBP
TaVRN-A1(85-191)-HIS
(c)
58
46
17
11
TaANR1a-MBP
TaVRN-A1(139-191)-HIS
(d)
58
46
17
11
TaANR1a-MBP
TaVRN-A1(85-179)-HIS
(e)
Ratio of TaANR1 binding protein with TaVRN1b to TaVRN1a
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
*
TaANR1-TaVRN-A1a
TaANR1-TaVRN-A1b
A.A.(85-91)
TaANR1-TaVRN-A1a
TaANR1-TaVRN-A1b
A.A.(139-191)
TaANR1-TaVRN-A1a
TaANR1-TaVRN-A1b
A.A.(85-179)

Figure 6. *In vitro* interactions between MADS proteins. *Ta*ANR1 is a protein with MBP-tag, and *Ta*VRN-A1 and *Ta*AGLG1 are the proteins with HIS-tag. (a) The protein interaction of *Ta*ANR1a (60.1 kDa) and Jagger *Ta*AGLG1a protein (23.79 kDa) or 2174 *Ta*AGLGb (23.78 kDa). Diamonds represent an area where no interacting proteins are observed between the *Ta*ANR1a and *Ta*AGLG1 proteins. (b) The protein interaction of *Ta*ANR1a with *Ta*VRN-A1(85–191) including both Leu^{117}/Phe^{117} and Ala^{180}/Val^{180} substitutions. The interacting proteins of *Ta*ANR1a with the 2174 *Ta*VRN-A1b (15.8 kDa) indicated by a star are stronger in protein band intensity than the interacting proteins of *Ta*ANR1a with the Jagger *Ta*VRN-A1a (15.7 kDa) indicated by a triangle. (c) The protein interaction of *Ta*ANR1a with *Ta*VRN-A1 (139–191) including Ala^{180}/Val^{180} substitution only. The interacting proteins of *Ta*ANR1a with the 2174 *Ta*VRN-A1b (9.3 kDa) indicated by a star are stronger in protein band intensity than the interacting proteins of *Ta*ANR1a with the Jagger *Ta*VRN-A1a (9.3 kDa) indicated by a triangle. (d) The protein interaction of *Ta*ANR1a with *Ta*VRN-A1 (85–179) including Leu^{117}/Phe^{117} substitution only. The interacting proteins of *Ta*ANR1a with the 2174 *Ta*VRN-A1b (14.5 kDa) indicated by a star are similar in intensity than the interacting proteins of *Ta*ANR1a with the Jagger *Ta*VRN-A1a (14.4 kDa) indicated by a triangle. At least three independent replicates were performed for each of these interactions. M indicates a protein marker. (e) Comparison of interacted *Ta*ANR1 proteins between *Ta*VRN-A1a and *Ta*VRN-A1b proteins.

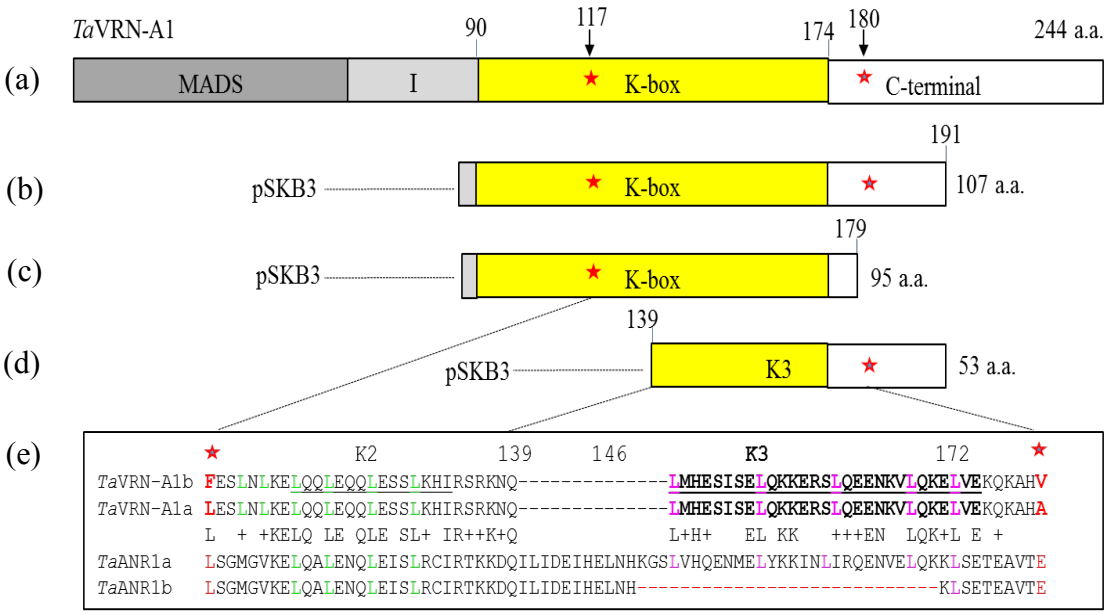


Figure 7. Interacting site of *Ta*VRN-A1 and *Ta*ANR1a proteins. (a) The locations of the conserved domains in *Ta*VRN-A1 and two altered site in the amino acids are indicated in red star. (b) *Ta*VRN-A1 fragment covering both Leu^{117}/Phe^{117} and Ala^{180}/Val^{180} substitutions was expressed to test differential interactions with *Ta*ANR1a. (c) *Ta*VRN-A1 fragment covering only Leu^{117}/Phe^{117} substitution was expressed to test differential interactions with *Ta*ANR1a. (d) *Ta*VRN-A1 fragment covering only Ala^{180}/Val^{180} substitutions was expressed to test differential interactions with *Ta*ANR1a. (e) Protein sequence alignment of the *Ta*VRN-A1a from Jagger, *Ta*VRN-A1b from 2174, and *Ta*ANR1a from Jagger. Conserved Leu residues are highlighted in green in the K2 box and in pink in the K3 box. The dash in red indicate missed amino acids due to a deletion in gDNA in *TaANR1b*.

Next, *Ta*VRN-A1 and *Ta*ANR1a were confirmed to interact with each other *in vivo* using bimolecular fluorescence complementation (BiFC). When *Ta*ANR1a was cloned into a pEG101-YFP vector and the construct transformed into tobacco leaves, enriched yellow fluorescent signals of *Ta*ANR1a-YFP were detected predominantly in the nucleus (Figures 8b-8d). The same pattern was observed for *Ta*VRN-A1 (Li et al., 2013). When *Ta*VRN-A1a was cloned into a pEG201-YN vector, and *Ta*ANR1a was cloned into a pEG202-YC vector, and these constructs were simultaneously expressed in the same cell, yellow fluorescence was again observed in the nucleus, confirming the *in vivo* interactions between these two proteins (Figures 9a–9d). The differential interactions of *Ta*VRNA1a and *Ta*VRN-A1b with *Ta*ANR1a provided initial evidence that *TaVRN-A1* might be the better candidate for *TaNUE1*.

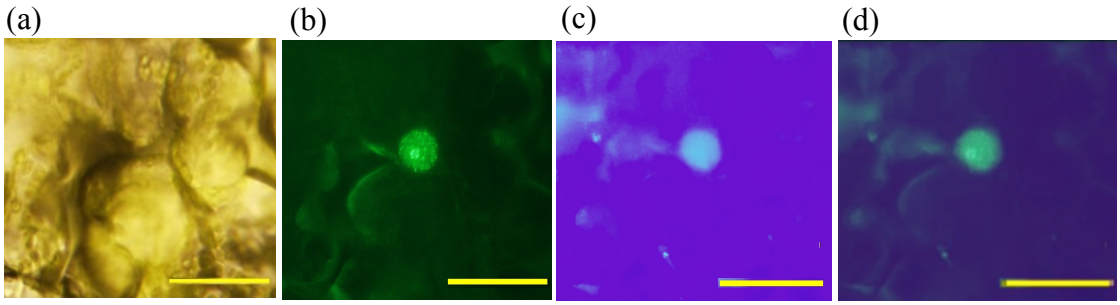


Figure 8. The subcellular location *Ta*ANR1-YFP protein in living cells of tobacco leaves. The *Ta*ANR1-YFP was expressed by pEG101 predominantly in a living cell. (a) Image of the *Ta*ANR1a protein under a fluorescent microscope with a bright filter (BF). (b) Image of the *Ta*ANR1a protein under a fluorescent microscope with a green filter. (c) Image of nucleus with 4′,6-diamidino-2-phenylindole (DAPI). (d) The overlay images for the alignment of the *Ta*ANR1a protein with the DAPI-stained nucleus. The scale bar in all images is 50 µm.

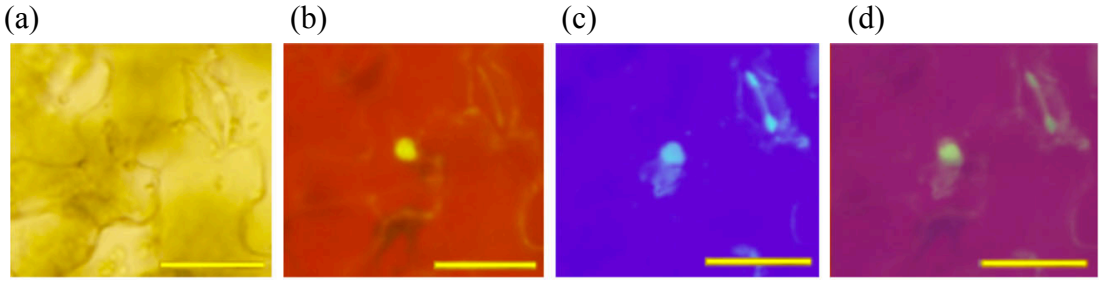


Figure 9 *In vivo* interactions of *Ta*VRN-A1 with *Ta*ANR1. *In vivo* interaction between *Ta*VRN-A1a-pEG201-YN and *Ta*ANR1a-pEG202-YC proteins. The paired proteins were simultaneously expressed in a living cell in *Nicotiana tabacum* (tobacco) leaves. (a) Image of the interacting proteins under a fluorescence microscope with a bright filter (BF). (b) Image of the interacting proteins under a fluorescence microscope with a green filter. (c) Image of the nucleus stained with 4',6-diamidino-2-phenylindole (DAPI). (d) Overlay images for the alignment of the interacting proteins with the DAPI-stained nucleus. The scale bar in all images is 50 μm.

Regulation of *TaVRN-A1* in normal and transgenic wheat plants

A link between *TaVRN-A1* and N regulation was discovered not only via its protein interaction with *Ta*ANR1, but also through its expression being regulated by N. In a separate experiment, four RILs carrying the Jagger *TaVRN-A1a* allele were tested in the same Kirkland soil and the same commercial soil. When N fertilizer was applied to plants grown for 11 weeks without fertilizer, transcription of the *TaVRN-A1a* allele was greatly down-regulated after 3 weeks (Figure 10a).

Next, the RNA interference (RNAi) approach was used to disrupt *TaVRN-A1a* expression in Jagger, and two individual transgenic plants (T20 and T40) were successfully generated. Transgenic T_1 populations were tested in the same Kirkland soil used to identify *QNue.osu-5A*. Transcription of *TaVRN-A1a* was greatly reduced in the positive transgenic plants compared with nontransgenic plants (Figure 10b). Furthermore, the down-regulatory effects of *TaVRN-A1a* by RNAi were reflected in the heading date-for nontransgenic plants, this was 130 days, while it was 138 days for the positive transgenic plants (Figure 10c). In the positive plants, transcription of *TaVRN-A1a* was further downregulated by N, compared with unfertilized positive plants (Figure 5b). After N fertilization, the heading date of positive transgenic plants was delayed to 143.4 days (Figure 10c). When T_2 populations of T20 (Figure 10d) and T40 (Figure 10e) lines were tested in the commercial soil without vernalization, a typical positive plant carrying *TaANR1::RNAi* demonstrated delayed heading, fewer fertile tillers and decreased grain yield, compared with either the nontransgenic plants or the wild-type Jagger.

Down-regulation of *TaVRN-A1* transcripts by N was also observed in six of seven winter wheat cultivars/lines that were naturally vernalized under field conditions. Vernalized plants were moved into a greenhouse where nutrients, temperature and light were controlled. Transcription of *TaVRN-A1* significantly decreased 3 weeks after application of N (Figure 10g). Down regulation of *TaVRN-A1* (Figure 10f) and delayed heading and increased biomass by N (Figure 10h) were also observed in spring wheat cv. Bobwhite.

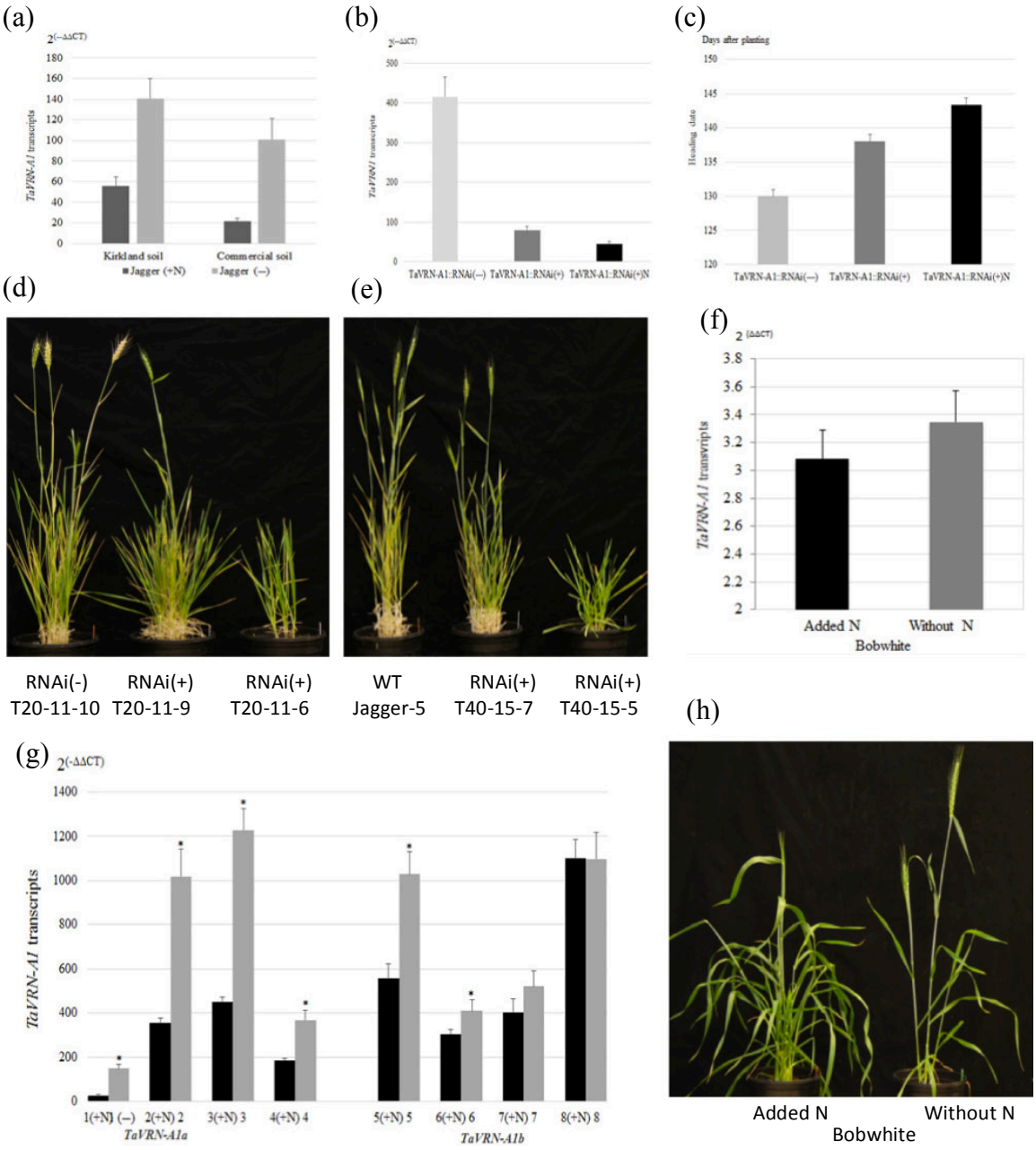

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
TaVRN-A1 transcripts
Kirkland soil
Commercial soil
Jagger (+N)
Jagger (–)
TaVRN-A1:RNAi(–)
TaVRN-A1:RNAi(+)
TaVRN-A1:RNAi(+)N
Days after planting
Heading date
Added N
Without N
Bobwhite
RNAi(-) T20-11-10
RNAi(+) T20-11-9
RNAi(+) T20-11-6
WT Jagger-5
RNAi(+) T40-15-7
RNAi(+) T40-15-5
1(+N) (–)
2(+N) 2
3(+N) 3
4(+N) 4
5(+N) 5
6(+N) 6
7(+N) 7
8(+N) 8
TaVRN-A1a
TaVRN-A1b

Figure 10. Regulation of *TaVRN-A1* by N in normal and transgenic wheat plants. (a) Regulation of *TaVRN-A1* transcript levels by N in Jagger grown in two different soils. (b) *TaVRN-A1* transcript levels in *TaVRN-A1::RNAi* transgenic Jagger wheat grown in the Kirkland soil. (c) Heading date of transgenic Jagger wheat grown in the Kirkland soil. *TaVRN-A1::RNAi*(+) indicates positive transgenic plants, whereas *TaVRN-A1::RNAi*(-) indicates nontransgenic plants. (d) Comparison of typical transgenic positive plants carrying *TaVRN-A1::RNAi*(+), and nontransgenic plants of the T20 line at the heading stage. (e) Comparison of a typical transgenic plant carrying *TaANR1::RNAi* of the T40 line, and wild-type Jagger plants at the heading stage. (f) Effects of N on *TaVRN-A1* transcript level in the spring wheat cultivar Bobwhite. (g) Effects of N on *TaVRN-A1* transcript level in eight vernalized winter wheat cultivars/lines. The dark columns indicate that N was utilized, whereas the grey columns indicate without N. 1. Jagger; 2. OK12716R/W; 3. OK11D25056; 4. Bentley, 5. Duster; 6. Gallagher; 7.Ruby Lee; 8. IBA; (+N) indicates that N was applied to the plants. Cultivars 1-4 carry the *TaVRN-A1a* allele, whereas cultivars 5-8 carry the *TaVRN-A1b* allele. (h) Effects of N on heading date in the spring wheat cultivar Bobwhite. Gene transcript levels were calculated using the $2^{(-\Delta\Delta CT)}$ method, where CT is the threshold cycle. Primers for *TaVRN-A1* and actin used as an endogenous control are provided in Table 7. The values represent mean expression levels (n = 7-16), and the bar indicates standard error. Asterisk indicates that the difference was significant between the two alleles ($Pr < 0.05$).

Function of *TaANR1* in a natural mutant and transgenic wheat

Two pieces of evidence confirmed that the *TaANR1* orthologue is also regulated by N in wheat. Firstly, a natural mutant of *TaANR1* was found to have a genetic effect on wheat development and growth. When conserved primers for homoeologous *TaANR1* genes were designed to test expression profiles, *TaANR1* transcripts were found not visibly in leaves but predominantly in roots in both Jagger and 2174. The PCR product was 250-bp in size in Jagger, but showed an additional band besides the 250-bp band in 2174 (Figure 11a). Sequence analysis indicated that the cDNA products with the single band from

Jagger were a mixture of homoeologous *TaANR1* transcripts from chromosome 2A (TGACv1_scaffold_093384_2AL) and chromosome 2D (TGACv1_scaffold_159992_2DL). In 2174, the cDNA products of the upper band were from *TaANR1* on chromosome 2D, and the cDNA products of the lower band were from *TaANR1b* onchromosome 2A; however, the cDNA fragment of the *TaANR1b* gene was 166-bp in size because the 84-bp exon 6 was missing. To determine whether this missing exon 6 was caused by an exon-skipping event, or by a deletion event at the gDNA level, the gDNA products of this gene were cloned and sequenced. Results showed that the lack of exon 6 in its cDNA was caused by a 23-bp deletion event comprising 10-bp at the 5′ end of intron 5 and 13-bp of exon 6 in the gDNA sequence of 2174 (Figure 11b). This included the AG splice site at the 5′ end of intron 5, resulting in the loss of the full 84-bp exon 6 in its mRNA, and thus 28 amino acids. These missing amino acids were included in the interaction site of *Ta*ANR1 with *Ta*VRN-A1 (Figure 7).

When phenotypic data of the RIL population used to map *QNue.osu-5A* were analyzed for genetic effects on *TaANR1*, there was a difference of 5.2 days in plants grown in commercial soil and 3.9 days in plants grown in Kirkland soil between the two alleles (Figure 11c). This result indicated that the Jagger *TaANR1a* gene is a minor heading repressor in wheat. The effect of *TaANR1* on grain yield in the RIL population tested in the field was in Chapter IV.

Three individual transgenic Jagger plants with *TaANR1::RNAi* were successfully generated, and their T_1 populations were tested in the commercial soil. *TaANR1* transcription was down-regulated in positive plants compared with nontransgenic plants, and *TaANR1* transcription was downregulated by N in the positive transgenic plants and

nontransgenic plants (Figure 11d). Compared with nontransgenic plants, the heading date of transgenic plants was 2.5 days earlier—this confirms that *TaANR1* has a minor and repressive effect on heading. A typical positive plant carrying *TaANR1::RNAi* showed an earlier developmental stage but fewer tillers during the juvenile phase compared with a nontransgenic plant (Figure 11e).

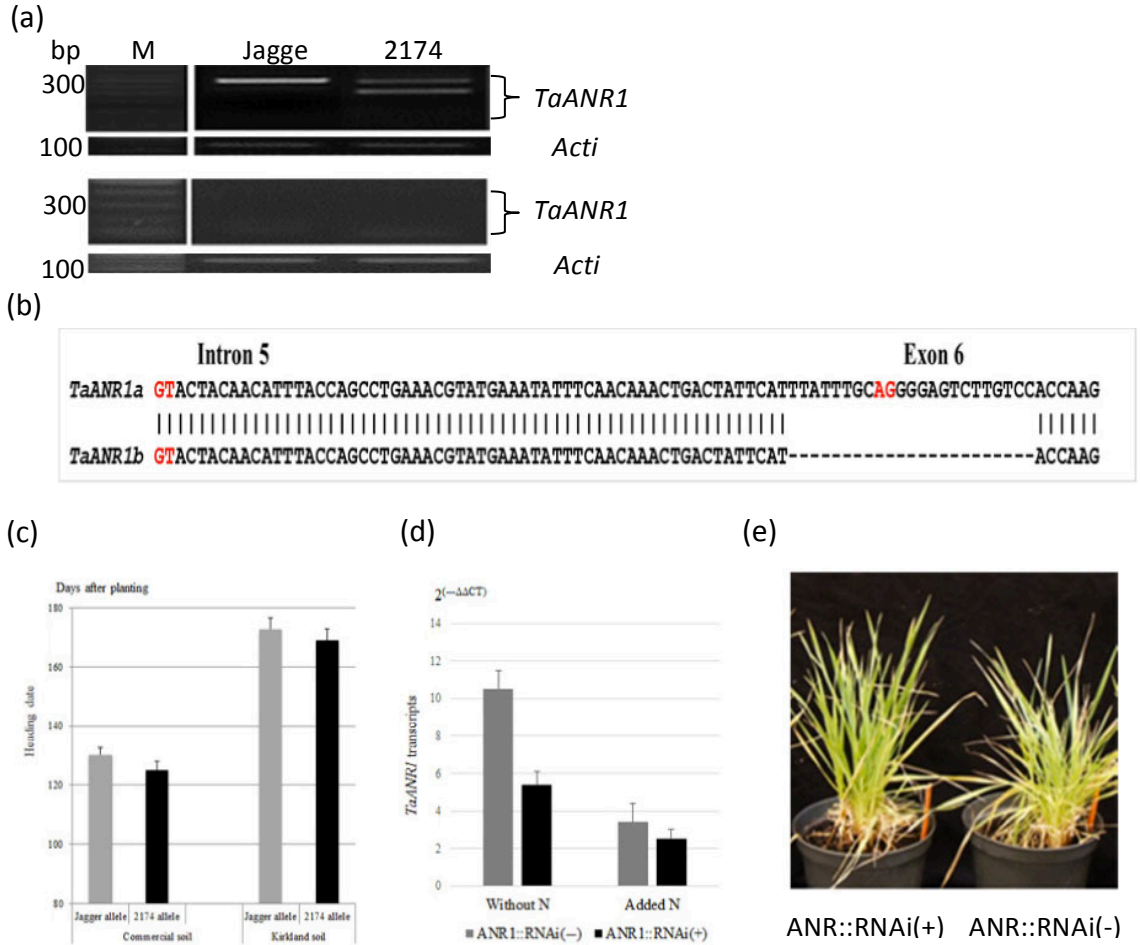


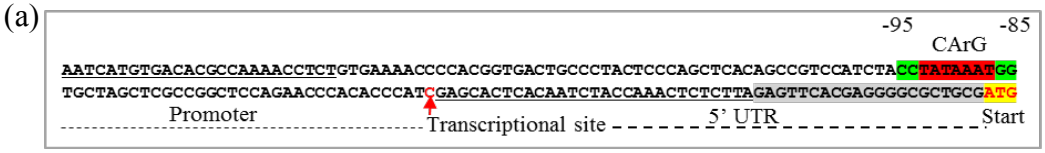
Figure 11. Function of *TaANR1* in natural mutant and transgenic plants. (a) Comparison of *TaANR1* expression patterns in root and leaf samples between Jagger and 2174. Primers ANR-MS-F2 (5'-GAATGGGTGTCAAGGAACTGCAGG-3') and ANR-MS-R2 (5'-GGAGTTCTTGAATTTCGGTTAACTTCAGTCA-3') were designed to amplify the 250-bp Jagger allele and the 166-bp 2174 allele. (b) A diagram of 23-bp indel in locations and sequences of *TaANR1* between the Jagger and 2174 alleles. The splicing sites at 5' end (GT) and 3' end (AG) of intron 5 are highlighted in red. (c) Genetic effect of *TaANR1* on heading date of RILs in the commercial soil and Kirkland soil. (d) Regulation of *TaANR1a* transcripts by N and RNAi transgenic wheat. Transcript levels were determined using values calculated by the $2^{(-\Delta\Delta}$CT) method, where CT is the threshold cycle. The values represent mean expression levels (n = 8), and the bars indicate standard errors. (e) Comparison of a typical transgenic plant carrying *TaANR1::RNAi* and nontransgenic plant at the juvenile stage.

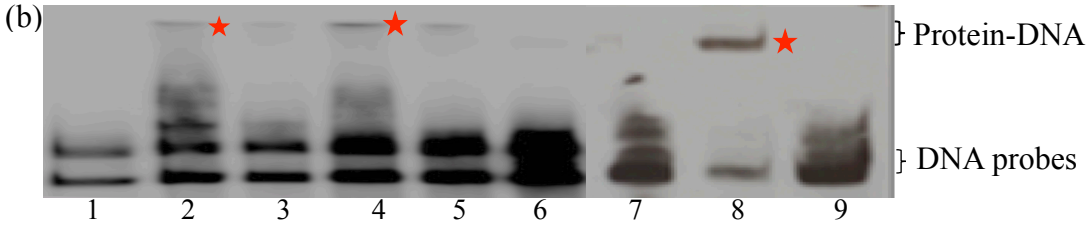
A direct interaction of *Ta*VRN-A1 with CArG box in the promoter of *Ta6SFT1* in the C metabolism

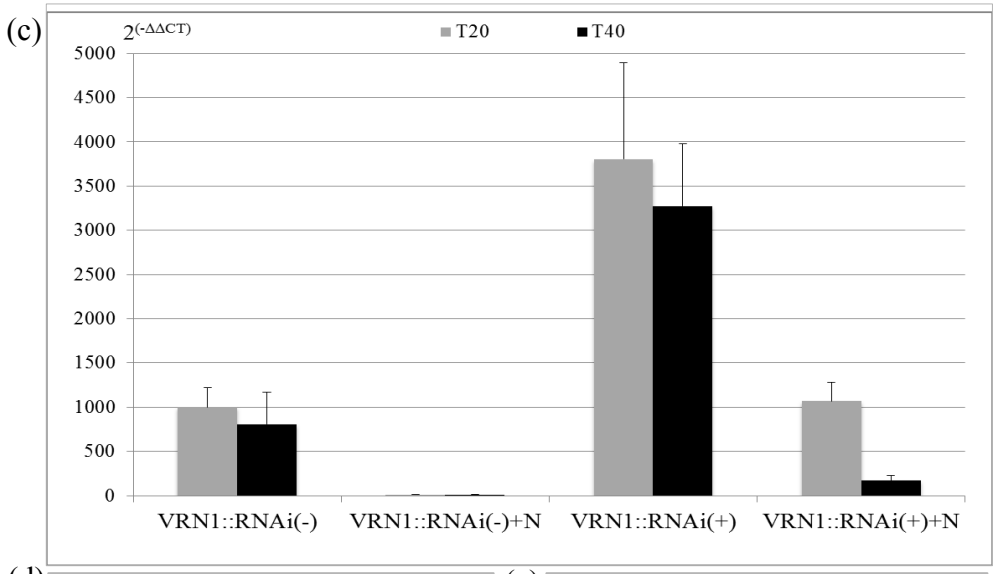
TaVRN-A1 is a transcription factor with a MADS-box domain that binds to the promoter of a gene to regulate the expression of said gene, and previous studies have indicated that the conserved DNA sequences of CArG box is a target of MADS-box proteins in wheat (Yan et al., 2003; Kane et al., 2005). An analysis found a CArG-box (CCTATAAATGG) present 85-95 bp upstream of the start codon of *Ta6SFT1*, a gene encoding sucrose:fructan 6-fructosyltransferase (Figure 12a). When temperate cereal plants such as wheat and barley were exposed to N-deficiency followed by N-resupply, *6SFT* was up-regulated by N-deficiency but was down-regulated during N-resupply (Duchateau et al., 1995). *Ta*VRN-A1 proteins encoded by both *TaVRN-A1a* and *TaVRN-A1b* alleles were found to have direct interactions with the *Ta6SFT1* promoter containing the CArG box using electrophoretic mobility shift assay (EMSA) (lanes 2 and 4, Figure 12b). Although *Ta*ANR1 protein also has a MADS-box domain, it showed little interaction with the *Ta6SFT1* promoter (lane 6, Figure 12b).

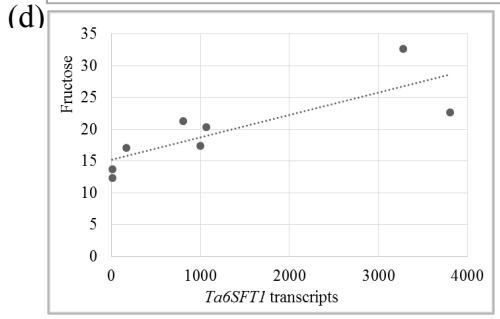
The transcript levels of *Ta6SFT1* in Jagger were determined using the same RNA samples as tested for *TaVRN-A1* and the qRT-PCR approach. Results showed that *Ta6SFT1* transcripts were dramatically down-regulated by N in normal plants (Figure 12c), indicating that N was a repressor to *Ta6SFT1*. In the *TaVRN1*::RNAi transgenic plants, *Ta6SFT1* transcripts were significantly increased (Figure 12c), indicating that *TaVRN-A1* was also a repressor to *Ta6SFT1*. When *TaVRN-A1* was interfered, the repressor was removed; hence, the transcript level of *Ta6SFT1* was increased. The consequence of the paradoxical effects of N and RNAi on *TaVRN-A1* resulted in decreased *Ta6SFT1* transcripts in the *TaVRN1*::RNAi transgenic plants with N compared with the negative plants without N (Figure 12c). When N was supplied to the *TaVRN1*::RNAi transgenic plants, *Ta6SFT1* was decreased due to the repression of N, but *Ta6SFT1* was simultaneously increased due to the repressive effect of N on *TaVRN-A1*. These results suggested that increased *Ta6SFT1* due to interference of *TaVRN-A1* did not negate the repressive effect from N.

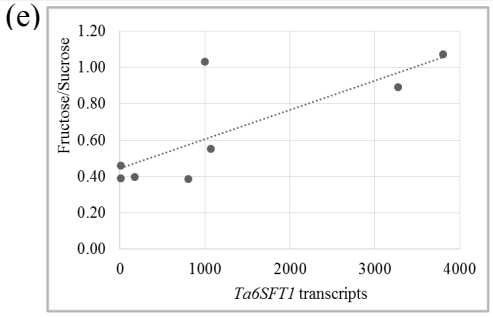
Further analyses were performed to determine how sugar content in leaves was regulated by changed transcript levels in normal wheat and transgenic plants. The *Ta6SFT1* transcript level positively correlated with fructose content ($r = 0.812$, $p < 0.05$) (Figure 12d) and the ratio of fructose/sucrose in leaves ($r = 0.79$, $p < 0.05$) (Figure 7e), whereas the ratio of fructose/sucrose was negatively correlated with sucrose in leaves ($r = -0.749$, $p < 0.05$) (Figure 12f). With the increase of the *Ta6SFT1* transcript level, fructan content in leaves was significantly increased ($r = 0.582$, $p < 0.05$) (Figure 12g). When *Ta6SFT1* was up-regulated in interfering *TaVRN-A1* in transgenic wheat, more fructose but less sucrose was accumulated, resulting in more fructans in leaves.

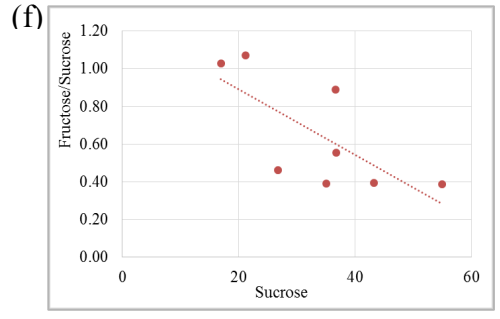

(a)
-95
-85
CArG
AATCATGTGACACGCCAAAACCTCTGTGAAAACCCCACGGTGACTGCCCTACTCCCAGCTCACAGCCGTCCATCTACCTATAAATGG
TGCTAGCTCGCCGGCTCCAGAACCCACACCCATCGAGCACTCACAATCTACCAAACTCTCTTAGAGTTCACGAGGGGCGCTGCGATG
Promoter
Transcriptional site
5' UTR
Start


(b)
Protein-DNA
DNA probes
1
2
3
4
5
6
7
8
9


(c)
2(-ΔΔCT)
T20
T40
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
VRN1::RNAi(-)
VRN1::RNAi(-)+N
VRN1::RNAi(+)
VRN1::RNAi(+)+N


(d)
Fructose
Ta6SFT1 transcripts


(e)
Fructose/Sucrose
Ta6SFT1 transcripts


(f)
Fructose/Sucrose
Sucrose

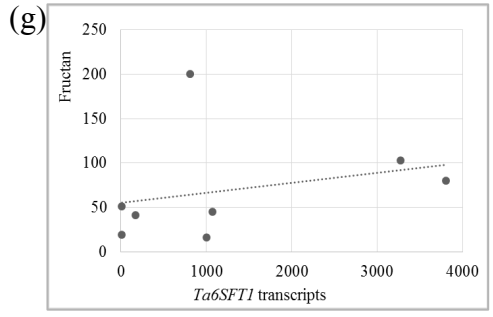

(g)
Fructan
Ta6SFT1 transcripts

Figure 12. Regulation of sugar content by the interaction of *Ta*VRN-A1 protein with the promoter of *Ta6SFT1*. (a) A diagram of CArG box in the promoter of *Ta6SFT1*. (b) EMSA analysis on interactions of *Ta*VRN-A1 protein with the *Ta6SFT1* promoter. The interacted product of protein and DNA is indicated by a star. 1) Biotin-ENBL *Ta6SFT1* probe but no *Ta*VRN-A1 protein; 2-3) *Ta*VRN-A1a MBP tag protein; 4-5) *Ta*VRN-A1b MBP tag protein; 2) and 4) Biotin-ENBL probe plus *Ta*VRN-A1 MBP tag protein; 3) and 5) Biotin-ENBL probe plus *Ta*VRN-A1 MBP tag protein but with 100×ENBL cold competitor. No or weaker interacting protein-DNA complex appears after the cold competitor was added. 6). Biotin-ENBL probe plus *Ta*ANR1 MBP tag; 7-9) A positive control for protein-DNA interaction provided by the kit. Four replicates were performed. (c) Regulation of *Ta6SFT1* transcript level in the *TaVRN1::RNAi* transgenic plants. Transcript levels were determined using values calculated by the $2^{(-_{\Delta\Delta}CT)}$ method, where CT is the threshold cycle. The values represent mean expression levels (n=9-20), and the bars indicate standard errors. Leaf samples were collected from T_1 progenies of two transgenic lines T20 and T40. The primers for *Ta6SFT1* and actin are provided in Table 7. (d) Positive correlation between *Ta6SFT1* transcripts and fructose content. (e) Positive correlation between *Ta6SFT1* transcripts and the ratio fructose/sucrose content. (f) Negative correlation between sucrose content and the ratio fructose/sucrose content. (g) Positive correlations between *Ta6SFT1* transcripts and fructans in leaves. (d-g) The values were averaged from three replicates, and the correlation analyzed (n=12).

3.4 Discussion

All the three candidate genes for *TaNUE1*, including *TaVRN-A1*, *TaAGLG1* and *TaCYB5* for *TaNUE1* were isolated and sequenced. In this study, experimental evidence clearly demonstrated that *TaVNR-A1* was *TaNUE1*. Firstly, the segregated NUE and related agronomic traits in RILs were associated with three candidates at the *TaVRN-A1* locus, and the *TaVRN-A1* gene was the best candidate responsible for the traits. Secondly, the Ala^{180}/Val^{180} substitution in the *Ta*VRN-A1 protein of two winter wheat cultivars caused

differential interactions with *Ta*ANR1, establishing a connection between the vernalization gene and the N-regulated gene. The function of *TaANR1* in wheat was validated by the down-regulation of *TaANR1* transcripts by N, and the delayed effect of *TaANR1* on heading date by RNA interference in transgenic plants and by analysis of a natural mutant. Lastly, RNA interference and N showed similar effects in the transgenic wheat in delaying heading date. However, precise effects of disrupted *TaANR1* expression on NUE need to evaluate in a field experiment.

Nitrogen has paradoxical effects on development. Lines started from seed and grown in the commercial soil showed accelerated plant development compared with those grown in the Kirkland soil. However, when N was supplied to the plants prior to stem elongation, the plant heading date was delayed. Plants accelerate the transition to the reproductive phase and flower faster with lower nutrients (Martinez-Zapater et al., 1994). This study indicated that when N was used to fertilize a plant prior to stem elongation, *TaVRN-A1* transcripts were down-regulated, and the plant heading date was thus delayed. This explains the molecular mechanism underlying the observation that how plants flower faster in N-deficient conditions. In winter wheat, *TaVRN-A1* expression is induced by vernalization, light or plant age (Danyluk et al., 2003; Li et al., 2013; Murai et al., 2003; Trevaskis et al., 2003; Yan et al., 2003). Without vernalization, *TaVRN-A1* is expressed in spring wheat because of the presence of multiple mutation forms, including indels of different forms in the promoter and the first intron (Dubcovsky et al., 2006; Fu et al., 2005; Pidal et al., 2009; Yan et al., 2003, 2004), a retrotransposon insertion in the 5′ UTR (Chu et al., 2011), an miRNA binding site in a miniature inverted repeat transposable element in the promoter (Yu et al., 2014) or the binding site of *TaGRP2* in intron 1

(Kippes et al., 2015). This study demonstrated that N regulates *TaVRN-A1* and flowering in both spring wheat and winter wheat cultivars.

The down-regulation of *Ta6SFT1* by the direct interactions of *Ta*VRN-A1 with the *Ta6SFT1* promoter provided evidence that *TaVRN-A1* is also involved in the C metabolic pathway. *Ta*6SFT1 is an enzyme catalyzing the formation and extension of β-2,6-linked fructans, which is typical of grasses (Sprenger et al., 1997). Silencing of *TaVRN-A1* using the RNAi approach, up-regulated *Ta6SFT1* transcript level was up-regulated in transgenic wheat, indicating that *TaVRN-A1* acted as a repressor in regulating *Ta6SFT1* expression. With the increase of *Ta6SFT1* by the interference of *TaVRN-A1* transcript accumulation, fructose content was increased but sucrose content was decreased, resulting in the accumulation of more fructans in leaves. The temporarily stored fructans are used in protecting winter wheat against environmental stresses like N deficiency, drought, and low temperature (Kawakami and Yoshida, 2005; Ruuska et al., 2008). The unraveling of interactions of *Ta*VRN-A1 protein with *Ta*ANR1 protein in the N absorption pathway and DNA elements in C metabolism pathway has laid the foundation of our understanding on NUE and provided an entry point for us to construct the gene/protein networks that regulate NUE in wheat.

CHAPTER IV

MARKER ASSISTED SELECTION FOR INCREASED NUENITROGEN USE IN WHEAT BREEDING

The major results in this study have been published in Plant Biotechnology,* ***Lei Lei*[1]*, Genqiao Li*[1]*, Hailin Zhang, Carol Powers, Tilin Fang, Yihua Chen, Shuwen Wang, Xinkai Zhu, Brett F. Carver, Liuling Yan, 2018. 16 (6): 117-123. ([1]these authors contributed equally to the project). I would like to express my deep thanks to all of the authors for their contributions to the research article.*

Abstract

Dual-purpose winter wheat in the Southern Great Plains requires more N due to the grazing in the winter season. Thus, developing new wheat cultivars with high NUE in low N scenarios is required. Marker assisted selection is helpful in the wheat breeding for pyramiding multiple genes for higher NUE into a novel cultivar. In our previous study, we reported that *Ta*HOX1, a homeobox protein involved in wheat heading date and development, had interaction with *Ta*VNR-A1 protein. In this study, we found that *Ta*HOX1 was competed binding to Ala^{180}/Val^{180} position of *Ta*VRN-A1 with *Ta*ANR1. Based on a 23-bp indel, a molecular marker for *TaANR1*

was also developed. Allelic variation in each of *TaVRN-A1*, *TaANR1*, and *TaHOX1* was used to determine genotypes of the RIL mapping population. When the RILs were tested in in adequate N condition in the field for two years, *TaVRN-A1a* and *TaANR1a* had a promotion effect on grain yield, whereas *TaHOX1a,* as a competitor with *Ta*ANR1, had a repressive effect on grain yield. Genetically incorporating favorable alleles from *TaVRN-A1a*, *TaANR1a*, and *TaHOX1b* increased grain yield from 9.83% to 11.58%. Markers for *TaVRN-A1*, *TaHOX1*, and *TaANR1* can be used for pyramiding the favorable alleles of higher NUE and grain yield in breeding of novel germplasm and wheat cultivars.

4.1 Introduction

Wheat is traditionally divided into two types: winter wheat that requires the plant to be exposed to low temperatures during a winter season to trigger its transition from vegetative to reproductive development (vernalization), and spring wheat, which has no requirement for vernalization (Pugsley, 1971). Compared to spring wheat, winter wheat requires significantly more N to achieve maximum grain yield, because it has a longer growing season with greater potential for N leaching, volatilization and run-off losses (Goos and Johnston, 1999). Dual-purpose winter wheat cultivars planted in the Southern Great Plains (USA) require more N, because the N is removed in grazed forge during the winter season (MacKown and Carver, 2007). Thus, developing new cultivars with higher NUE is critical when limited N fertilizers are supplied to winter wheat.

QTL mapping is an effective approach to find the gene associated with the desired phenotype. Once the function of the gene that regulates the QTL is characterized, molecular markers can be developed based on the polymorphisms between two alleles and used in for marker assisted selection. With the help of QTL mapping, some genes

related to N regulation and NUE have been identified, such as *GS1* and *GOGAT* in wheat (Habash et al. 2007; Quraishi et al. 2011).

Gene pyramiding is the process of combining several favorable genes together into a single genotype. This is possible through conventional breeding, but phenotyping of different traits often is not easy in certain population types such as F_2 populations. Thus, DNA markers can be used to genotype different genes that represent different traits without phenotyping. Pyramiding has been mostly applied in combining multiple disease resistance genes in previous studies. For example, pathogens that frequently overcome single gene host resistance due to the emergence of new plant pathogen races, it is desirable to develop a cultivar with multiple resistance genes that can provide broad-spectrum resistance (Shanti et al. 2001). Three genes that are resistant to a major disease, bacterial blight of rice, were pyramided using marker-assisted selection and the genes in combinations were found to provide high levels of resistance to seven different races of the pathogen (Singh et al. 2001). In our study, NUE is a polygenic trait and is not usually determined by a single gene. Pyramiding different alleles for high NUE will facilitate the breeding process to develop a new cultivar with increased grain yield. In this study, we found that *TaVRN-A1a*, *TaANR1a*, and *TaHOX1b* alleles had an effect on increasing grain yield. By pyramiding these alleles, it is possible to develop a novel wheat cultivar for increased N use and high grain yield.

4.2 Material and Methods

Developing PCR markers for *TaANR1*

Two alleles of *TaANR1* were sequenced. A 23-bp deletion was found in '2174' allele. Markers were developed based this deletion. The specific primers are listed in Table 7.

In vitro protein interactions

One cDNA of *TaHOX1a* and *TaHOX1b* encoding proteins from 1 to 150 a.a., were each cloned into a pSKB3 vector with an N-terminal 6×HIS-tag (Li et al., 2013). The cDNAs encoding proteins from 111 to 240 a.a. (end) for *Ta*ANR1a, and from 111 to the end for *Ta*ANR1b, were cloned into a pMAL-c2X vector with an MBP-tag (New England BioLabs, Ipswich, MA, USA). The primers used for cloning are listed in Table 7. Constructs were expressed in E. coli (BL21 DE3). An Ni-NTA column (Qiagen, Germantown, MD, USA) was used to purify the expressed proteins from pSKB3 with the 6×HIS-tag, and an amylose column (New England BioLabs) was used to dialyse and purify the protein fused with the MBP-tag. The protein fused with the 6×HIS-tag (500 μg/mL, 50 μL) was incubated in an Ni-NTA column (40 μL) for 1 h at 4 °C in binding buffer with 10 mM imidazole, and the MBP-tag protein (500 μg/mL, 100 μL) was then added to the column. MBP-tag protein (500 μg/mL, 100 μL) alone was added to a Ni-NTA column as a negative control for nonspecific interactions between MBP-tag and the Ni-NTA column. Reactions were incubated overnight at 4 °C. The interacting proteins in the resin were then washed with chilled binding buffer with 35 mM imidazole six or eight times until the MBP-tag protein disappeared in the negative control. The protein was eluted by boiling the samples in 40 μL of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer for 5 min. The eluted protein samples (5 μL) were analyzed on a 12% SDS-PAGE gel stained with Coomassie Brilliant Blue. When the protein fused with the MBP-tag was incubated in the amylose column, the 6×HIS-tag

protein was added to the amylose column and then washed with column buffer until the 6×HIS-tag protein disappeared in the negative control. Interacting proteins were analyzed in pull-down assays.

In vivo protein interactions

The complete cDNA of *TaANR1a* and the cDNA fragment of *TaHOX1a* encoding its protein from 1 to 150 a.a. were cloned into pDONR207 with the BP Cloning Kit (Invitrogen) according to the manufacturer's instructions. The LR Cloning Kit (Invitrogen,Waltham, MA, US) was then used to transfer *TaANR1* to pEarleygate 101 (pEG101) for subcellular localization of *Ta*ANR1a. Primers used for constructs are provided in Table 7. Previous reports located *Ta*HOX1 in the nucleus of the cell (Li et al., 2013). The *TaANR1* in pDONR207 was fused to the C-terminal amino acid portion (175–239 a.a.) of YFP in the pEarleyGate202-YC vector (pEG202-YC) to test *in vivo* interactions with *Ta*HOX1 (1–150 a.a.), which was fused to the N-terminal 174 amino acid portion (1–174 a.a.) of YFP in the pEarleyGate201-YN vector (pEG201-YN). Reciprocal empty vectors were also used as negative controls for interactions with *Ta*ANR1 and *Ta*HOX1 proteins. *Agrobacterium tumefaciens* strains (GV3101) carrying the BiFC constructs and p19 strain were used together to infiltrate *Nicotiana benthamiana* leaves 5 weeks after planting. Leaves were ready for BiFC imaging after 3 days of infiltration. Images were visualized under a BX-51 fluorescence microscope (Olympus). Images were taken with a bright filter (BF) to indicate the background of the leaves infiltrated with *A. tumefaciens* carrying constructs, or with an ultraviolet filter to indicate the position of the nucleus stained with 4', 6-diamidino-2-phenylindole (DAPI),

and with GFP and GFPA filters to indicate the presence of fluorescent proteins. Scale bars in all images are 50 μm.

Testing the RIL population in the field

Yield experiments on 96 RILs of the Jagger × 2174 population were conducted for 2 years. The first experiment took place at the North Central Agronomy Research Station near Lahoma, OK, and the population was planted as a standard yield plot on October 30, 2007. The plot area (3 m×1.26 m) was fertilized before planting according to soil-test recommendations for adequate N to attain a yield goal of approximately 3000–6700 kg/ha in this specific site (Edwards et al., 2012). The second experiment was conducted at the Agronomy Research Station in Stillwater, OK, and the population was planted as a head-row plot on 14 November 2014. At the experiment site, the top 30 cm of soil contained 40 ppm N, 16 ppm P_2O_5 and adequate K with a pH of 5.3. Diammonium phosphate (DAP, 8-5-14) fertilizer of 309 kg/ha and urea of 371 kg/ha were incorporated into the soil by cultivator tillage. The experimental design was a randomized complete block with two replications. Dry grains for each plot were weighed to determine grain yield.

4.3 Results

Development of a PCR marker for *TaANR1*

Based on the 23-bp indel (Figure 11b), a polymorphic PCR marker for *TaANR1* was developed (Figure 13). The natural *TaANR1b* mutant allele exists in 13 of 69 wheat cultivars across the USA, but 12 of the 13 *TaANR1b* wheat cultivars were utilized in the Southern Great Plains (Table 5).

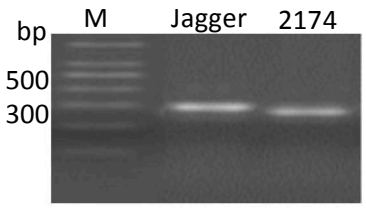


Figure 13. A PCR marker for 23-bp indel between the Jagger *TaANR1a* allele and the 2174 *TaANR1b* allele. Primers ANR-MF1 (5'-ATCACAAGGTACTACAACATTTAC-3') and ANR-MR1 (5'-GGAGTTCTTGAATTTCGGTTAACTTCAGTCA-3') were designed to amplify the Jagger allele (286 bp) and the 2174 allele (263 bp). M: DNA marker.

Allele		Source Cultivar (with source of mapping population)
TaANR1a	CAP	UC1110 and CIMMYT-2 (PI 610750) (CA), Rio Blanco (ID), Zak and ID0556 (ID), P91193 and P92201 (IN), Heyne and KS01HW163-4 (KS), GRN*5/ND614-A and NY18/CC 40-1 (MN), McNeal and Thatcher (MT), Reeder/Bw-277 "R" Entry#5 and Reeder/Bw-277 "S" Plants G+LL (ND), Cayuga and Caledonia (NY), Pio 25R26 and Foster (NY), Stephens and OR9900553 (OR), Finch and Eltan (WA), Louise and Penawawa (WA), Clark's Cream and CIMMYT III (CIGM90.250-2), Weebill and Jupateco (CIMMYT), Platte and CO940610 (CO), SS550 and PIONEER 26R46 (GA), TAM105 (NE).
	SGP	Jagger, Custer, Cutter, Doans, Duster, Endurance, Fannin, Intrada, Jagalene, Jei110, Lakin, Overley, Protection, TAM 112, Danby, Deliver, Fuller, Guymon, Neosho, OK Bullet, Santa Fe, TAM 111.
TaANR1b (mutant)	CAP	IDO444 (ID), Harry and Wesley (KS).
	SGP	2174, Above, Centerfield, Hatcher, Ripper, OK102, OK Field, Shocker, TAM 110, Trego.

Table 5. Wheat cultivars used for determining the frequency of *TaANR1* alleles.

CAP: Coordinated Agriculture Project. SGP: Southern Great Plains.

*Ta*ANR1 and *Ta*HOX1 bound to the Ala^{180}/Val^{180} position of *Ta*VRN-A1

The Ala^{180} in the *Ta*VRN-A1a protein was found to be critical for maintaining the ability to interact with *Ta*HOX1, a homeobox protein involved in wheat heading date, and the mutated Val^{180} in *Ta*VRN-A1b decreased the ability to bind with *Ta*HOX1 (Li et al., 2013). In this study, the Ala^{180} in the *Ta*VRN-A1a protein was less able to interact with *Ta*ANR1a than the mutated Val^{180} in *Ta*VRN-A1b (Figures 9b–9d). These results suggest that *Ta*VRN-A1, *Ta*ANR1 and *Ta*HOX1 might form a protein complex in wheat. To test whether *Ta*ANR1 interacts with *Ta*HOX1, these two proteins were analyzed for *in vitro* and *in vivo* interactions.

No significant interaction was detected between *Ta*ANR1a and *Ta*HOX1 *in vitro* (Figure 14). However, *Ta*ANR1a interacted with *Ta*HOX1 *in vivo* (Figures 15b–15d). This indicated that *Ta*ANR1a and *Ta*HOX1 bound to the Ala^{180}/Val^{180} position of *Ta*VRN-A1. The Jagger *Ta*HOX1a protein has a leucine residue at position 99 that is substituted with a proline residue in the 2174 *Ta*HOX1b protein. Both *Ta*HOX1a and *Ta*HOX1b showed *in vivo* interactions with *Ta*ANR1a. These results suggest that the differential interactions between *Ta*VRN-A1, *Ta*ANR1 and *Ta*HOX1 proteins might modify N-related traits.

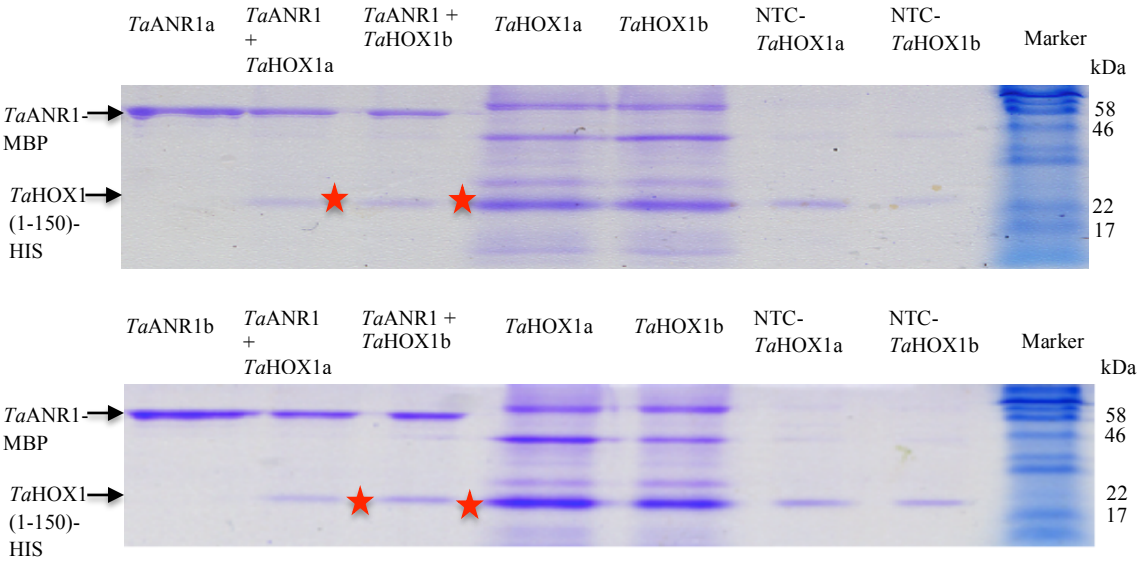


Figure 14. *In vitro* interaction of *Ta*HOX1 and *Ta*ANR1a or *Ta*ANR1b proteins. *Ta*HOX1a from Jagger (20.3 kDa), *Ta*HOX1b from 2174 (20.3 kDa), *Ta*ANR1a from Jagger (60.1 kDa), and *Ta*ANR1b from 2174 (56.7 kDa) were expressed *in vitro*. The protein in the position where a star is placed is not from interaction because the protein intensity is similar as that in the negative controls (NTC-*Ta*HOX1). At least three independent replicates were performed for each of these interactions.

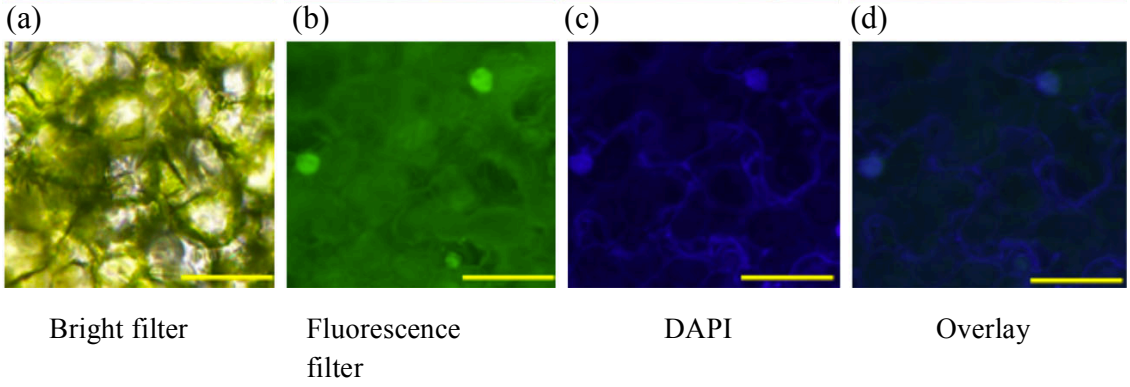


Figure 15. *In vivo* interaction of *Ta*ANR1 with *Ta*HOX1. *In vivo* interaction between *Ta*HOX-A1a-pEG201-YN and *Ta*ANR1a-pEG202-YC proteins. The paired proteins were simultaneously expressed in a living cell in *Nicotiana tabacum* (tobacco) leaves. (a) Image of the interacting proteins under a fluorescence microscope with a bright filter (BF). (b) Image of the interacting proteins under a fluorescence microscope with a green filter. (c) Image of the nucleus stained with 4',6-diamidino-2-phenylindole (DAPI). (d) Overlay images for the alignment of the interacting proteins with the DAPI-stained nucleus. The scale bar in all images is 50 μm.

Genetic effects of *TaVRN-A1*, *TaANR1* and *TaHOX1* on grain yield in the field

To evaluate the combined effects of favorable alleles of *TaVRN-A1*, *TaANR1* and *TaHOX1* on grain yield, we tested the effects of natural mutations on grain yield in the 96 RIL population under field conditions for 2 years and in two locations. This approach circumvented regulatory roadblocks associated with cultivating genetically modified crops.

When tested in the field at high N levels in the 2007–2008 growing season, lines carrying the Jagger *TaVRN-A1* allele achieved a higher grain yield by an average of 2.82% compared with plants carrying the 2174 *TaVRN-A1* allele (Figure 16a). The Jagger

TaVRN-A1 allele increased grain yield by 2.92% in the 2014–2015 growing season, when a population of the same RIL was grown in a field with sufficient N to attain high grain yield (Figure 16b). The *TaANR1a* in Jagger was observed to increase grain yield. Lines carrying the Jagger allele achieved higher grain yields by an average of 3.39% (2.8% in the 2007–2008 growing season, and 3.97% in the 2014–2015 growing season), compared with plants carrying the 2174 allele (Figures 16a, 16b). *TaHOX1a* had a repressive effect on grain yield. Lines carrying the Jagger *TaHOX1a* allele achieved a lower grain yield by a decrease of 4.46% from 4.69% on average in the 2007–2008 growing, and 4.22% in the 2014–2015 growing season compared with plants carrying the 2174 *TaHOX1b* allele (Figures 16a, 16b). Together, *TaVRN-A1a* and *TaANR1a* in Jagger and *TaHOX1b* in 2174 significantly increased grain yield by an average of 10.71% (9.84–11.58%) ($Pr < 0.05$), based on the average grain yield of the 96 RILs tested in the field for 2 years and in two locations.

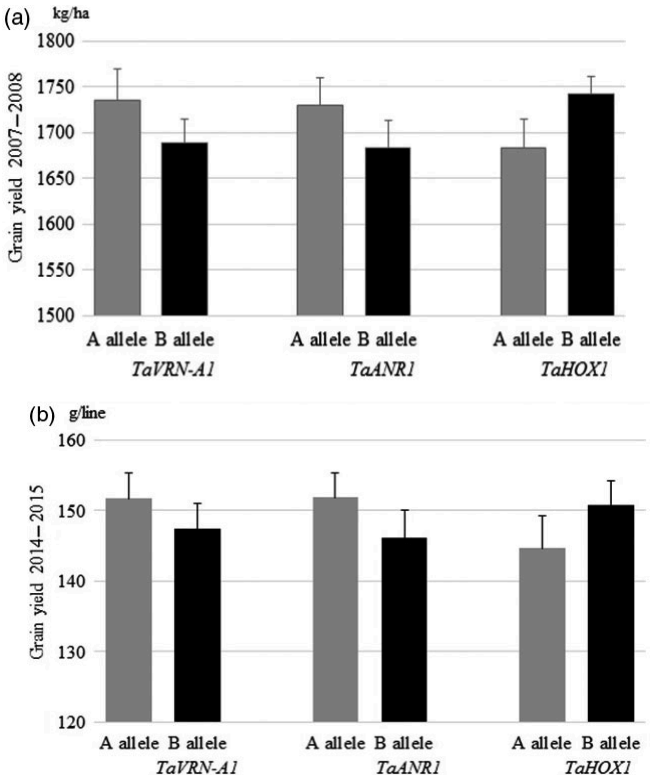


Figure 16. **Genetic effects of *TaVRN-A1*, *TaANR1* and *TaHOX1* on grain yield.** (a) Genetic effect of three genes on grain yield in recombinant inbred lines (RILs) tested in the field in the 2007–2008 growing season. (b) Genetic effect of three genes on grain yield in RILs tested in the field in the 2014–2015 growing season. The average values of each gene in the population of 96 Jagger × 2174 RILs were compared for two alleles: 'A' for those lines (n = 25-58) carrying the Jagger allele, and 'B' for those lines (n = 48-61) carrying the 2174 allele. The bars indicate standard errors.

4.4 Discussion

*Ta*HOX1 is a homeobox protein involved in the regulation of vernalization duration for winter wheat by having differential interaction with *Ta*VRN-A1a and *Ta*VRN-A1b proteins, and the mutated Val^{180} in *Ta*VRN-A1b decreased the ability to bind with *Ta*HOX1 (Li et al., 2013). In this study, the cloned *TaVRN-A1* allowed for the

identification of its interactors in the same pathway. The *in vivo* interaction between *Ta*ANR1 and *Ta*HOX1 suggested that *Ta*VRN-A1, *Ta*ARN1, and *Ta*HOX1 might form a protein complex and the differential interactions between these three proteins might modify N-related traits. It is a critical finding that *Ta*ANR1 and *Ta*HOX1 competed to bind to the Ala^{180}/Val^{180} position of *Ta*VRN-A1.

The competition between *Ta*ANR1 and *Ta*HOX1 for *Ta*VRN-A1 is reflected in their effects on grain yield. A total of 96 RILs were tested in the field under adequate N condition for two years (2007-2008 growing season and 2014-2015 growing season). As a competitor with *Ta*ANR1 for *Ta*VRN-A1, *Ta*HOX1a had a repressive effect on grain yield. Lines carrying the Jagger *TaHOX1a* allele achieved a lower grain yield by a decrease of 4.46% compared with plants carrying the 2174 *TaHOX1b* allele. Both *TaVRN-A1a* and *TaANR1a* had a promoting effect on grain yield. On the contrary, RILs carrying the Jagger *TaVRN-A1a* allele obtained an average higher grain yield by 2.87% compared with plants carrying the 2174 *TaVRN-A1b* allele. Similarly, RILs carrying the Jagger *TaANR1a* allele achieved higher grain yields by an average of 3.39% compared with plants carrying the 2174 *TaANR1b* allele. These genes caused subtle modifications to grain yield in a common high-N scenario in the field, but genetically incorporating favorable alleles of the *TaVRN-A1a* and *TaANR1a* genes from Jagger, and the *TaHOX1b* gene of 2174, increased grain yield by an average of 10.71% (9.84% to 11.58%).

Based on the results in this study, molecular marker assisted selection by pyramiding genes can be applied to enhance NUE. In the previous study, markers have already been developed for two genes of *TaVRN-A1* and *TaHOX1* (Li et al., 2013). In this study, we developed the marker for identifying two alleles from *TaANR1* based on a 23-bp deletion

event comprising 10-bp at the 5′ end of intron 5 and 13-bp of exon 6 in the gDNA sequence of 2174 exon. All of these molecular markers could be applied during the selection of favorable alleles of genes in the breeding programs.

CHAPTER V

DISCUSSIONS AND CONCLUSIONS

5.1 *QNue.osu-5A* was discovered associated with NUE

Wheat is one of the most important crops worldwide. With the increasing human population, the challenges for the next decades will be to accommodate the needs of the expanding world population by building a highly productive agriculture. Application of N fertilizer is the most direct and efficient way to obtain the higher yield in crops. Studies for optimizing N usage in wheat have already been done by scientists through soil and fertilizer management. Developing wheat varieties with high NUE is a good strategy to improve N use via genetic improvement.

In this study, a major QTL (*QNue.osu-5A*) for N-related agronomic traits has been identified using a population of recombinant inbred lines of winter wheat under contrasting N fertilization regimes. *QNue.osu-5A* explained a large part of the total phenotypic variation in several N related agronomic traits. For instance, it accounted for 38.5% and 55.3% of the total variation in grain yield, when the population was grown

under N deficient soil and then fertilized with two contrasting N fertilizer (100N and 25N).

In cereal crops, NUE refers to the ratio of grain yield to N supplied by soil and fertilizer, which is dissected into two components: N uptake (NUpE) and N utilization efficiency (NUtE) (An et al., 2006; Moll et al., 1982). In this study, we used grain yield to describe NUE. The allelic effect of *TaNUEa* on grain yield was increased to up to 140% when the population was grown in the Kirkland soil with limited N in the greenhouse without vernalization. The allelic effect of *TaNUEa* on grain yield was increased up to 18.1% when the critical recombinant lines with limited genetic backgrounds were grown under low-N conditions in the field with natural vernalization. The enhanced effects observed in plants grown under low-N conditions facilitated positional cloning of *TaNUE1*. Three candidate genes including *TaVRNA-1*, *TaAGLG1,* and *TaCYB5* were identified as candidates for *TaNUE1* causing *QNue.osu-5A*.

5.2 *TaVRN-A1* was the final candidate gene for *TaNUE1*

In previous studies, *TaVRN-A1* was cloned as a gene for variations in the qualitative vernalization requirement between spring wheat and winter wheat (Yan et al., 2003) and for the quantitative vernalization requirement between weak winter wheat and strong winter wheat using a positional cloning approach (Li et al., 2013). In addition to the known function in vernalization, *TaVRN-A1* locus had been reported to be associated with variation in stem elongation in winter wheat Jagger x 2174 RIL population (Chen et

al. 2009), which supports that *TaVRN-A1* locus has the pleiotropic effect on wheat development.

In this study, *TaVRN-A1* has been identified as the *TaNUE1,* which also supports the pleiotropic effect of the gene. Firstly, *Ta*VNR-A1 had direct interaction with *Ta*ANR1. The differential interaction between two alleles of *Ta*VNR-A1 with *Ta*ANR1 was caused by the Ala^{180}/Val^{180} substitution in the predicted K-box region. *ANR1* was the first identified gene from MADS-box gene family with known function as a key regulator of lateral root growth in response to signals from external NO_3^- in Arabidopsis (Zhang and Forde, 1998). *TaANR1* is the wheat orthologue of *ANR1* in Arabidopsis and the functions of *TaANR1* in wheat were characterized by discovering the natural mutant and generating transgenic wheat. From the direct interaction, we can believe that *TaVRN-A1* was involved in N regulation pathway through interaction with *Ta*ANR1. Secondly, the expression of both *TaVNR-A1* and *TaANR1* was down-regulated by N, further supporting that *TaVRN-A1* was involved in N regulation pathway. Thirdly, the function of *TaVRN-A1* and *TaANR1* was characterized in the transgenic wheat by disrupting *TaVRN-A1* or *TaANR1* through RNAi approach. The heading date was delayed and fewer tillers were observed in the *TaVNR-A1* positive transgenic plants. *TaANR1* had a minor repressive effect on the heading date and a typical transgenic plant carrying *TaANR1::RNAi* also showed reduced plant size which indicted *TaANR1* functioned in plant development and N metabolism pathways. Fourthly, *Ta*VRN-A1 was involved in the sugar metabolism pathway through the interaction with promoter of *TaSF6F1*. *TaVNR-A1* is likely to be involved not only N metabolism pathway but also in carbon assimilation pathway.

TaSF6F1 is also a key component in regulating the grain yield and carbohydrate synthesis in wheat.

Three homoeologous genes, *TaVRN-A1*, *TaVRN-B1*, and *TaVRN-D1*, exist in common wheat. In this study, only allelic variation in *TaVRN-A1* was found to link with the segregated NUE and related agronomic traits in the winter wheat population, and only *Ta*VRN-A1 was tested for its interaction with *Ta*ANR1. The homoeologous *TaVRN-B1* and *TaVRN-D1* and their proteins should also have similar effects on the N-related agronomic traits, although the role of the genes in the N-related agronomic traits under different genetic backgrounds needs to be investigated.

5.3 Increasing grain yield by pyramiding favorable alleles of *TaVRN-A1*, *TaANR1,* and *TaHOX1*

Our previous study demonstrated that vernalization duration of winter wheat was affected by the differential interaction between two alleles from *Ta*VRN-A1 and *Ta*HOX1 (Li, et al. 2013). In this study, we show the *Ta*ANR1 have direct interaction with *Ta*HOX1 *in vivo*. Therefore, *Ta*VRN-A1 may compete with *Ta*HOX1 in the vernalization pathway and *Ta*ANR1 in the N metabolism pathway. The competition between *Ta*ANR1 and *Ta*HOX1 for *Ta*VRN-A1 is reflected in their effects on grain yield. *TaHOX1a* showed a repressive effect on grain yield, which functions as a competitor for *Ta*ANR1 in N regulation pathway, whereas *TaANR1a* and *TaVRN-A1a* had a positive effect on grain yield. By using molecular markers to select the favorable alleles of genes with high NUE, for example, alleles of *TaVRNA1a*, *TaANR1a*, and *TaHOX1b*, it is possible to develop a novel wheat cultivar with improved NUE and high grain yield.

5.4 Future research perspectives

Characterization of candidate genes using transgenic plant is an effective way to decipher its function, in which, genome editing has become the substitution for the widely used RNAi approach in disrupting gen function. In the CRISPR/Cas9 genome editing system, plants express an RNA sequence called guide RNA containing a designed hairpin structure that has gRNA targeting sequence ("spacer") and scaffold sequence, providing both targeting specificity and the binding ability for Cas9 nuclease (Joel McDade, 2015). The binding specificity of Cas9 with the target DNA is determined by both gRNA-DNA base pairing and a protospacer-adjacent motif (PAM) immediately downstream of the target region. Cas9 from different bacterial species requires different PAM sequences. Both nuclease domains of Cas9 (HNH and RuvC-like) cleave one strand of double-stranded DNA at the same site (three-nucleotide distance from the PAM) (Joel McDade, 2015). The edited site will create a small or large deletion in the genome sequence, which will be repaired often with some mistakes by DNA repair mechanism of the cell. DNA repair mistakes such as frame shift mutation in the coding sequence of a gene caused by an insertion or deletion of a base can lead to the loss of function by producing partial or non-functional protein. Compared with the approach of RNAi disrupting the gene in transcription level, the genome editing approach could disrupt the gene in the genomic sequence, which directly altered the structure of the expressed protein and that may cause it to lose its original function. A single transgenic plant carrying genome editing construct for *TaVNR1* in 2174 and one for *TaANR1* in Jagger background were generated (data was not shown). Further research is needed in using these two transgenic plants to analyze the response of other genes to N after disrupting these two genes. For instance, RNAseq

analysis for the pattern of gene expression could be used to find genes that are regulated by *TaVRN1* or *TaANR1* in N regulation pathway and studies could be focused on genes with changed expression pattern and their connections to the N regulation pathways.

Breeders may take advantage of the molecular markers that are associated with high NUE. With the information of molecular markers, it would help breeders to combine favorable alleles to develop wheat varieties that have high NUE. Similarly, overexpression of relevant gene is another approach for improving NUE. For example, enhancing N uptake could be gained by overexpression of nitrate and ammonium transporters. In future, breeding for high NUE cultivars will be highly possible by combining direct gene transfer together with the marker-assisted selection. This new generation of crops will help to solve the problem caused by the increasing cost of N fertilizer.

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APPENDICES

Table 6. Primers for gene expression

Primer name	Primer sequence (5'-3')*	Products
VRN1-Exp-F1	GAATAAAGTTCTCCAGAAGGAACTCGTG	104 bp
VRN1-Exp-R2	GCATGAAGGAAGAAGATGAAGAGCTG	
ANR-MS-F2	GAATGGGTGTCAAGGAACTGCAGG	250 bp
ANR-MS-R2	GGAGTTCTTGAATTTCGGTTAACTTCAGTCA	
Actin-F2	GGAACTGGCATGGTCAAGGCTG	107 bp
Actin-R2	CCCATCCCCACCATCACACC	

Table 7. Primers for protein-protein and protein-DNA interactions, and gene transformation

Primer name	Primer sequence (5'-3')[d]	Vector	Products
VRN1(85)*Nde*IF[a]	AGCAGCGG**CATATG**TCTGAAATTCAGGGAAACTG	pSKB3	85 to 191 a.a.
VRN1(191)*Bam*HIR[a]	AGGAGA**GGATCC**TCAGCTGGTTTGAGGCTGAG		
VRN1(85)*Nde*IF[a]	AGCAGCGG**CATATG**TCTGAAATTCAGGGAAACTG	pSKB3	85 to 179 a.a.
VRN1(179)*Bam*HIR[a]	AGGAGA**GGATCC**TCAATGGGCCTTCTGCTTCTCC		
VRN1(139)*Nde*IF[a]	GTGGT**CATATG**ATCAGATCCAGGAAGAACCAACTTATGCACG	pSKB3	139 to 191 a.a.
VRN1(191)*Bam*HIR[a]	AGGAGA**GGATCC**TCAGCTGGTTTGAGGCTGAG		
ANR-EcoRI(110)F[a]	CGGAA**TTCTTG**ATGGGACAAGATCTTTCTGGAATGG	pMAL-c2X	111 to end
ANR-BamHI(240)R[a]	AGGAGA**GGATCC**TCATGGATGTAGTTGCAATCCTAG		
AGLG1-NdeI-F1[a]	AGCAGCGG**CATATG**ATGGGTCGCGGCAAG	pSKB3	1 to 180 a.a.
AGLG1(180)-BamH-R1[a]	AGGAGA**GGATCC**CTATGTCGCCTCCAGCTCGA		
HOX1-full-NdeI-F[a]	AGCAGCGG**CATATG**GAGAGCGACTGCCAGTTCCTG	pSKB3	1 to 150 a.a.
HOX1(150)-BamHI-R1[a]	AGGAGA**GGATCC**CTAGCCGCCGCAGCTAGCG		
VRN1-BiFC2-F1[a]	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGGCGGGGGAAGG	pEG101 pEG201-YN	Full length
VRN1-BiFC2-R1[a]	GGGGACCACTTTGTACAAGAAAGCTGGGTCCCCGTTGATGTGGCTCAC		
ANR-BiFC2-F1[a]	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGGCGCGGCAAGATAGTG	pEG101 pEG202-YC	Full length
ANR-BiFC2-R1[a]	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGGATGTAGTTGCAATC		
HOX1-BiFC2-F1[a]	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAGAGCGACTGCCAGTTC	pEG101 pEG201-YN	1 to 150 a.a.
HOX1(150)-BiFC2-R1[a]	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCCGCCGCAGCTAGCG		
ANR(1-180)-EcoRI F1[b]	ATCCGGAATTCATGGGGCGCGG	pMAL-c2X	1 to 180 a.a.
ANR(1-180)-BamHI R1[b]	AGGAGAGGATCCTCATAACTCAACATT	pMAL-c2X	1 to 180 a.a.
VRN1(1-180)-EcoRI F1[b]	ATCCGGAATTCATGGGGCGGGGGA	pMAL-c2X	1 to 180 a.a.
VRN1a(1-180)-BamHI R1[b]	AGGAGAGGATCCTCACGCATGGGC	pMAL-c2X	1 to 180 a.a.
VRN1b(1-180)-BamHI R1[b]	AGGAGAGGATCCTCACACATGGGC	pMAL-c2X	1 to 180 a.a.
VRN1-Ri-F1[c]	GGACTAGTGGCGCGCCATGTATGGACAAAATTCTTG	pMCG161	536 bp
VRN1-Ri-R1[c]	TTATAAGCGATCGCCCTAGGCTCACCATCCACGGTGGAAG		
ANR-Ri-F1[c]	ACTAGTGGCGCGCCCCGGACAAAAAAGGACC	pMCG161	310 bp
ANR-Ri-R1[c]	GCGATCGCCCTAGGTGGATGTAGTTGCAATCCT		

[a] Primers for protein-protein interactions
[b] Primers for protein-DNA interactions
[c] Primers for cDNA fragments that were used for RNAi constructs
[d] Boldfaced sequences indicate the restriction enzyme sites for cloning

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